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Early Dissemination, Dormancy, and Metastasis

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14. ABSTRACT

In the last period before this final report, we devoted effort to both aims, to optimize methods and publish the first phase of efforts. **SA1** was to understand the intrinsic mechanisms of dissemination by early-progressed cancer cells and how the microenvironment in these primary sites, named P-TMEM (*Primary Tumor Microenvironment of Metastases*), contribute to early dissemination.

Our data suggested that a signature of early dissemination markers consisting on HER2^{HI}/p-ATF2^{LO}/E-cadherin^{LO} could identify motile early-progressed tumor cells and predict for dissemination. We further showed that mammary tissue macrophages and HER2^{HI}/p-ATF2^{LO}/E-cadherin^{LO} tumor cells cooperate to disseminate early. This further supports our hypothesis that even in early cancer lesions macrophages and tumor cells assemble with endothelial cells a TMEM structure. We also made progress in generating a standardized triple staining that captures all these cell types in all tissues and we used intravital imaging to document live intravasation in early cancer lesions. Our work revealed that the presence of intraepithelial macrophages in DCIS samples in mouse models or humans could accurately predict dissemination in 70% of the cases of patients with bone marrow DTCs (**Disseminated Tumor Cells**).

SA2 focused on elucidating how S-TMEMs (*Secondary Tumor Microenvironment of Metastases*) contribute to the dormancy phase of early DTCs. We found that early DTCs appear to undergo a p38-independent but TWIST-dependent dormancy in secondary organs, revealing a new pathway for dormancy induction. Our data also showed that depletion of macrophages during early dissemination steps significantly reduces metastasis that develop in the late stages of progression, providing functional support for macrophages in P- and S-TMEM structures in metastasis development. This work is part of two papers, one published in the journal *Nature* and the other published in *Nature Communications* (*accepted pending editorial revisions*). We also report the progress made in the sorting protocol to isolate CFP+/HER2+ DTCs from lungs and CTCs in the MMTV-Neu-CFP model, and that we have performed single cell RNA-seq which will allow us to identify the early and late gene signatures of DTCs that we proposed in our grant.

15. SUBJECT TERMS

dormancy, early dissemination, EMT, macrophages, TMEM

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INTRODUCTION

Our *hypothesis* proposed the existence of a *primary tumor microenvironment of metastasis* or P-TMEM, previously only detected in invasive cancers, in pre-malignant lesions. We proposed that this P-TMEM is required to induce EMT and early dissemination of ErbB2^{high}/P-p38^{low} MECs. We further hypothesized that in order to exit dormancy early DTCs need to maintain the *secondary*-TMEM (S-TMEM) after extravasation. If early DTCs cannot sustain the S-TMEM they then persist in dormancy. This is critical because even in patients bearing invasive lesions, early DTCs may constitute a large subpopulation of dormant DTCs that evade therapies. Our *rationale* was that by understanding early dissemination and dormancy we may find ways to eradicate dormant DTCs and prevent metastasis. Our original specific aims were:

AIM 1. To test how ErbB2^{high}/P-p38^{low} MECs assemble P-TMEM during early dissemination. Our *goals* were to 1)- use intravital imaging and MMTV-ErbB2-CFP, MMTV-PyMT-EGFP-c-fs-CFP transgenic mice and modulation of ErbB2 and p38 signaling to detect P-TMEM function during intravasation of “early” and “late” ErbB2⁺ MECs, 2)- obtain expression profiles from early and late CTCs to identify genes involved in MΦ recruitment and dormancy onset and 3)- perform a pilot analysis of human tissues to correlate early dissemination markers with DTCs in bone marrow of patients. We *expected* to identify how ErbB2⁺ MECs assemble P-TMEM, how early CTCs are predisposed to enter dormancy, and that human sample analysis would correlate early dissemination markers with the presence of DTCs in bone marrow.

AIM2. To test the role of S-TMEM in regulating early DTC dormancy. The majority of pre-malignant solitary early DTCs are dormant and not associated with MΦ. In contrast, MΦ are always embedded in proliferating metastasis even if small. Our *goals* were to **A)** test the function of S-TMEM during early DTCs dormancy and **B)** isolate early and late DTCs to determine their dormancy profiles and the mechanisms that distinguish their

S-TMEM assembling potential. We *expected* to identify pathways regulating dormancy of early and late DTCs and how these differ in their ability to induce metastatic-niches in target organs. We anticipated these data to reveal novel mechanisms to target dormant DTCs.

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Introduction: We provide a summary of the original aims above, their rationale and specific progress focused on the tasks set for this proposal. At the end of the Results we list the additional tasks that will be expanded but that are beyond the scope of this proposal. Of note is that the support from this DoD award has resulted in the publication of several papers in top tier journals focusing on microenvironmental regulation of metastasis initiation. Two papers in Nature^{1,2} identified mechanisms of early dissemination, dormancy and metastasis in breast cancer, one paper in Nature Cell Biology focused on hypoxia-regulated priming of DTCs for dormancy³ and a new paper that is now accepted pending editorial revisions in Nature Communications identified the role of macrophages in the establishment of primary TMEM in early lesions. A fourth paper⁴ focused on technological advancements fueled by the intravital imaging work performed under this grant. This shows that high level of productivity this grant has fueled.

Specific Aim 1- To test how ErbB2^{high}/P-p38^{low} MECs assemble P-TMEM during early dissemination. SA1.1. Objective: Use intravital imaging and MMTV-ErbB2-CFP, MMTV-PyMT-EGFP-c-fs-CFP transgenic mice and modulation of ErbB2 and p38

signaling to detect P-TMEM function during intravasation of “early” and “late” ErbB2⁺ MECs.

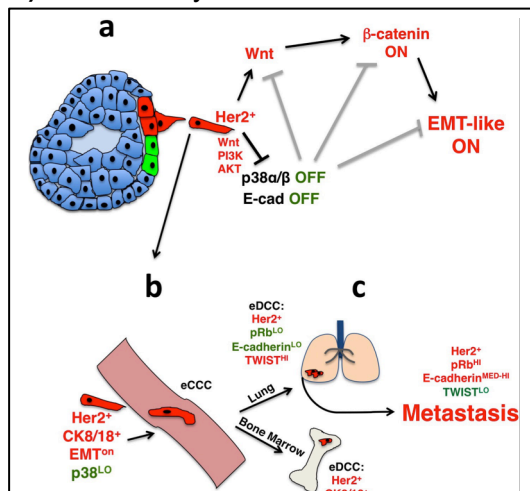


Fig.1: Cartoon depicting the mechanism of early dissemination by Her2⁺ early lesion cells. a) Early Her2⁺ early lesion cancer cells (red) turn on Wnt, PI3K and AKT signaling, inhibit p38 activation and E-cadherin-junction formation allowing for a Twist1^{HI} EMT-like invasive program; p38 and E-cadherin inhibit the Wnt- and β-catenin-driven EMT-like program and invasion (grey inhibitory symbols). **b)** Her2⁺ p38^{LO} Twist1^{HI} E-cad^{LO} early lesion cancer cells, which retain CK8/18 expression, can intravasate and disseminate. **c)** In lungs more than 85% of eDCCs (red) were Her2⁺ E-cad^{LO} (p-Rb or p-H3)^{LO}, suggesting a large population of dormant cells. Most eDCCs are also Twist1^{HI} E-cad^{LO}. Nevertheless, eDCCs can initiate metastasis, which correlated with the acquisition of a Twist1^{LO} E-cad^{MED-HI} phenotype. In the bone marrow, eDCCs were Her2⁺ CK8/18⁺ and remain dormant for the duration of the experiments, as bone lesions were never observed.

Results: We established and performed live imaging to follow the intravasation of early cancer cells using MMTV-Neu-CFP (cyan fluorescent protein) mice. We also detected the effect of p38 inhibition on early dissemination. See **Fig.1** for a graphic summary of our findings and see experimental details in the attached article (**Appendix 1**), published in *Nature*^{1,2}, where the movies are available .

We had also described how early dissemination from pre-invasive HER2+ lesions is driven by mammary gland tissue resident macrophages, one of the P-TMEM components. We completed these studies and submitted a manuscript to *Nature Communications* where it has been **accepted** pending editorial revisions (Appendix 2).

Briefly, we found that P-TMEMs (containing macrophages) can be found in pre-invasive HER2+ structures. These early macrophages induce a WNT-dependent EMT, supporting our novel finding that macrophage-driven dissemination occurs during these early breast cancer stages. Depletion of macrophages before overt tumor detection drastically reduced early dissemination and diminished the onset of metastasis even when macrophage depletion was stopped when tumors became invasive. Resident CCR2+/CD206+/VCAM-/Tie2+ macrophages were attracted into early lesions by CCL2 produced by early HER2+ cancer cells in an NFkB-dependent manner. Intra-epithelial macrophages and loss of E-cadherin junctions was also found in human DCIS, but not normal breast tissue. Interestingly the Tie2 is a marker of the macrophages that in large primary tumors open the endothelium allowing for the entry of tumor cells into the vasculature¹. Thus, it seems that a population of mammary resident macrophages during very early stages of cancer are executing the same function of TAMs in invasive tumors.

Conclusions: We confirmed that P-TMEM is formed in HER2-driven tumors. This already occurs in pre-malignant lesions in the absence of invasive cancer. We also conclude that macrophages are vital for early dissemination and that macrophages and early cancer cells are critical contributors to late metastasis.

SA1.2. Objective: Obtain expression profiles from early and late CTCs to identify genes involved in MΦ recruitment and dormancy onset.

Results: In order to isolate CTCs (also termed circulating cancer cells, CCCs) by FACS, we initiated the optimization of flow cytometric detection of tumor cells in the MMTV-ErbB2-CFP model. As reported in PR of FY1, our initial experimental plan to detect MMTV-HER2-CFP cancer cells based on their CFP expression was hampered by the fact that CFP could also be detected in other host cells not expressing HER2 and when looking for rare cells in circulation or target organs with low DTC burden this was difficult to achieve. In FY2, we therefore changed our approach and optimized flow cytometric analysis of the HER2 oncogene. We were able to successfully detect HER2+ early and late cancer cells both in the primary site and the lung as a secondary site. We then processed them using the 10x Chromium system and submitted these samples for RNAseq analysis. This effort was dedicated towards both SA1.2 and SA2.2 and is described in detail in SA2.2 below. We are currently awaiting results from the RNA seq analysis. Once we obtain the data, we will then analyze the expression of macrophage recruitment factors in early vs. late cancer cells to test the hypothesis that early cancer cells fail to attract macrophages and therefore fail to form S-TMEM and enter dormancy instead. Thus, we will have completed our aims in their totality.

Additionally, we had proposed that lack of association of macrophages with DTCs may foster dormancy while interaction with macrophages might stimulate awakening from dormancy or prevent dormancy all together. Models such as the MMTV-HER2 develop metastases mostly in the lung but never in the bone⁵. Yet, DTCs are frequently observed in the bone marrow^{2,5}. This raised the question whether bone resident macrophages have a different effect on DTC proliferation than lung resident macrophages. By comparing how bone marrow and lung macrophages function we may better understand how S-TMEMs function in different target organs. We investigated the direct effect of lung and BM resident macrophages

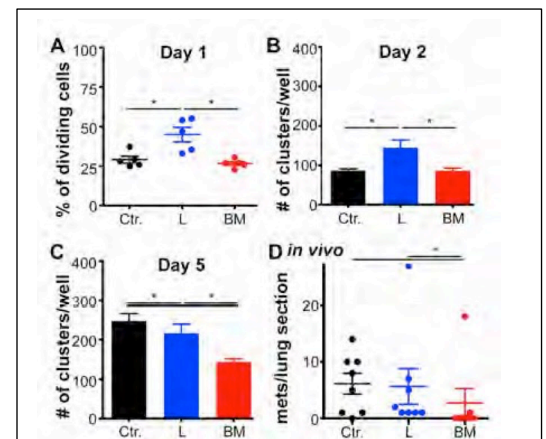
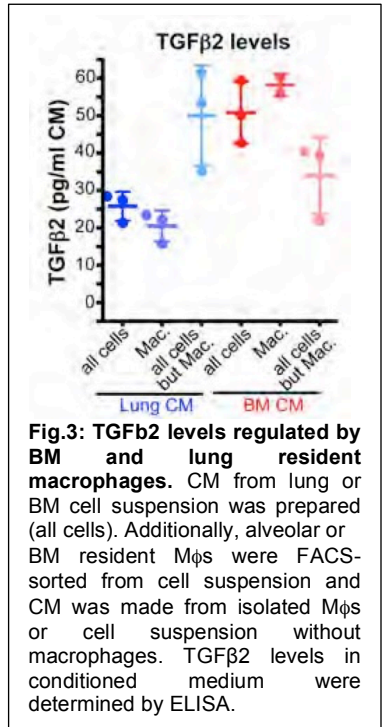


Fig.2: Direct effect of lung and BM macrophages on growth of 4T1 cells in 3D in vitro and in vivo. 4T1-GFP cells were seeded in matrigel either alone or mixed with primary lung or bone marrow MΦs. Dividing 4T1 cells were quantified after 1 day and clusters >2cells were quantified on day 2 and 5. While lung MΦs initially stimulated proliferation (**A,B**), BM MΦs had a long term growth inhibitory effect (**C**). **D**) 4T1-mCherry cells were injected into the tail vein of wild type mice alone or in a 1:1 ratio with either lung or BM resident MΦs. Metastasis per lung section was quantified.

on cancer cell proliferation by performing a direct 3D co-culture of primary alveolar and BM resident macrophages with the 4T1 cancer cell line in Matrigel (**Fig.2A-C**). We found that while lung macrophages initially stimulated proliferation (**Fig.2A,B**), BM macrophages had a long-term inhibitory effect on the proliferation of 4T1 breast cancer cells (**Fig.2C**). We then tested this possibility in an experimental metastasis assay *in vivo* and co-injected 4T1-mCherry cancer cells with either lung or BM resident Mφs purified via FACS. The addition of BM Mφs significantly reduced metastasis formation compared to co-injection with lung Mφs or injection of 4T1 cancer cells alone (**Fig.2D**). This led us to the conclusion that while lung resident macrophages initially stimulate proliferation, BM resident Mφs seem to have a long term growth inhibitory effect.

Because in previous work we had described that high levels of TGFβ2⁶ induce dormancy of DTCs in the bone microenvironment, we tested the production of TGFβ2 by resident Mφs from either lung or BM (**Fig.3**). We found that TGFβ2 levels were higher in total BM suspension and in primary BM Mφs CM compared to total lung and lung Mφ CM. Additionally, when Mφs were removed from the organ cell suspensions by FACS sorting (“all cells but mcs”), this significantly increased TGFβ2 levels in lung suspension and significantly reduced TGFβ2 levels in BM suspension. This argues that macrophages in lungs may suppress the formation of dormancy-inducing microenvironments and set the stage for testing how STEM in lungs function when macrophages are affected.



Conclusion: We successfully established the isolation of HER2+ cells from primary and secondary sites and were able to isolate RNA from isolated cells. This will allow us to identify factors in early vs. late DTCs that are responsible for Mφ recruitment (See below). We further identified that association of DTCs with Mφs is not sufficient to induce proliferation but the tissue context and macrophage subtype seem to be crucial as well. Specifically, while lung resident Mφs foster proliferation, BM resident Mφs seem to induce DTC dormancy due to the expression of high TGFβ2 levels. This might explain why in the bone even highly aggressive DTCs are not able to form metastases: upregulation of Mφ-recruiting factors and subsequent recruitment of bone resident Mφs would only result in a more stable state of dormancy in the bone microenvironment.

SA1.3. Objective: To correlate early dissemination markers in primary breast tumors (DCIS and invasive lesions) with DTC presence in BM.

Results: We were able to show the infiltration of human early stage breast cancer lesions (DCIS) by macrophages, and a decrease in E-cadherin levels associated within the vicinity of the macrophages (see **Appendix 2** for more details).

Conclusion: Both the identification of intra-epithelial macrophage numbers with E-Cadherin levels and the formation of P-TMEM structures in pre-invasive lesions seem to be a promising tool to predict early dissemination. We postulate that combining TMEM detection of early cancer cells with macrophages around the vasculature and E-Cadherin levels will provide a powerful tool for the prediction of early dissemination in DCIS patients.

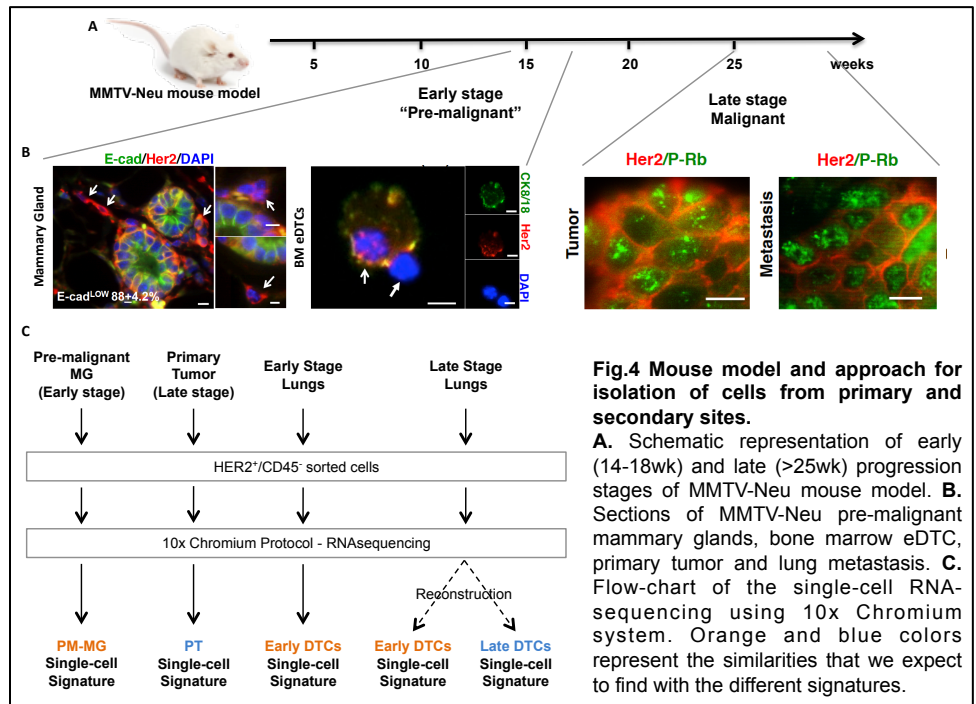
Specific aim 2- To test the role of S-TMEM in regulating early DTC dormancy. SA2.1. Objective: To test whether early DTCs form S-TMEM and if these influence escape from dormancy.

Results: Our aim proposed that the formation of S-TMEMs is required for the escape from dormancy and that early DTCs might be less efficient in forming S-TMEMs. Our data in **Appendix 1** clearly supports the notion that for prolonged periods DTCs remain in a dormant state and **Appendix 2** supports the function of macrophages during early dissemination.

SA2.2. Objective: Molecular profiling of early DTCs.

In order to recover early and late DTCs in target organs and identify the gene programs distinguishing their behavior we optimized a protocol for the isolation of single cells from MMTV-Neu mice by FACS sorting. As

indicated we experienced problems due to leakiness of the MMTV promoter in target organs (see SA1.2), thus to overcome this problem we optimized a FACS protocol specific for the Her2 oncogene expressed by the cancer cells and excluded the CD45 false positive cells. We also used FvB animals, both normal mammary glands and normal lungs, as negative controls for the oncogene. Given the low abundance of some of these DTCs we decided to incorporate single cell RNA-seq into our pipeline in order to take advantage of any cell we can recover. Given the dramatic evolutionary differences between early and late populations we are comparing, single cell analysis should reveal differences if we are able to compile the analysis of a few thousand cells, as well as some heterogeneity within DTCs in the same group. Moreover, we may be able to predict their origin in the case of late stages, in which there is a mixture of early and late DTCs. **Figure 4** shows the pipeline from cell sorting to RNA-seq we have utilized, taking advantage of the 10x Chromium system. Data from this experiment is currently being analyzed.



KEY RESEARCH ACCOMPLISHMENTS

Specific Aim 1

- Macrophages enter pre-invasive lesions before any overt tumors can be detected, and induce a canonical WNT-dependent EMT (Appendix 1) in cooperation with an intrinsic HER2-induced EMT program linked to non-canonical Wnt signals^{1,2}. Depletion of macrophages in MMTV-HER2 mice before overt tumor detection drastically reduces early dissemination and diminishes the onset of metastasis even when macrophage depletion was stopped when tumors became invasive. (Appendix 1)
- Early DTCs can found metastasis and ~80% of metastasis are derived from early DTCs and 20% in a linear fashion from primary tumors^{1,2}.
- Resident CCR2+/CD206+/VCAM-/Tie2+ macrophages are recruited into early lesions by CCL2 produced by early HER2+ cancer cells in an NFkB-dependent manner (Appendix 1).
- In collaboration with the Condeelis lab, we found that intra-epithelial macrophages inside early lesions in MMTV-HER2 mice are often associated with the vasculature and thus resemble P-TMEM structures (Appendix 1).
- Using *in vitro* and *in vivo* approaches, we found that addition of lung resident Mφs promote proliferation of 4T1 breast cancer cells, while addition of BM resident Mφs have an adverse growth-inhibitory effect, indicating that S-TMEM formation might have organ specific effects.
- We analyzed more DCIS lesions (N=12) and found that the association of intra-epithelial macrophages with reduced E-Cadherin levels was significant. (these findings are reported in Linde et al. 2016 – (Appendix 1)).
- Successfully performed intravital imaging and provided video evidence of intravasation during early stages of cancer progression when P-TMEM is forming in these tissues².

Specific Aim 2

- We optimized the FACS sorting of early HER2+ cancer cells as well as disseminated cancer cells from lungs of mice carrying early and late stage.HER2+ mammary cancer.

- Optimized the usage and isolation of single cells using the C1 Fluidigm system. Used this expertise to optimize the isolation of DTCs and profiling using the 10X Chromium system. Data should be available by the summer of 2018.
- Optimized the RNA extraction and library creation for single cell RNA-seq and successfully obtained RNA-seq data from ~14 single cells from early and late stages of progression using the FACS protocol for HER2.
- Identified gain of TWIST and loss of E-cadherin expression as a potential important regulator mechanism for early dissemination and DTC dormancy in lung².

REPORTABLE OUTCOMES

Publications relevant to this project:

1. Entenberg D, Pastoriza JM, Oktay MH, Voiculescu S, Wang Y, Sosa MS, Aguirre-Ghiso J, Condeelis J. Time-lapsed, large-volume, high-resolution intravital imaging for tissue-wide analysis of single cell dynamics. **Methods**. 2017 Sep 1;128:65-77. doi: 10.1016/j.ymeth.2017.07.019.PMID:28911733
2. Fluegen G, Avivar-Valderas A, Wang Y, Padgen M, Williams JK, Nobre AR, Calvo V, Cheung JF, Bravo-Cordero J, Entenberg D, Castracane J, Verkhusha V, Keely PJ, Condeelis J and Aguirre-Ghiso JA. Phenotypic heterogeneity of disseminated tumor cells is preset by primary tumor hypoxic microenvironments. **Nature Cell Biology**. 2017. Doi: 10.1038/ncb3465.
3. Entenberg D, Pastoriza JM, Oktay MH, Voiculescu S, Wang Y, Sosa MS, Aguirre-Ghiso JA, Condeelis J. Time-lapsed, large-volume, high-resolution intravital imaging for tissue-wide analysis of single cell dynamics. **Methods**. Vol 128, 1 Sept 2017, pages 65-77. ISSN: 1046-2023. Doi: 10.1016/j.ymeth.2017.07.019.
4. Kathryn Harper, Maria Soledad Sosa, Hedayatollah Hosseini, Alvaro Avivar Valderas, Chandandaneep Nagi, Roger Davis, Christoph Klein, David Entenberg, John Condeelis, Eduardo F. Farias and Julio A. Aguirre-Ghiso. Identification of an ErbB2+ early disseminating pre-malignant cancer cell subpopulation with metastatic potential. **Nature**. 2016 Dec 14. Doi: 10.1038/nature20609.PMID: 27974798.
5. Hedayatollah Hosseini, Kathryn Harper, Maria Soledad Sosa, Lahiri Kanth Nanduri, Christian Werno, Carolin Ehrl, Matthias Maneck, Milan Obradovic, Nina Patwary, Gundula Haunschild, Christian Reimelt, Florian Weber, Josef Schroeder, Julio Aguirre-Ghiso, Piero Musiani, Andreas Hartkopf, Florin-Andrei Taran, Tanja Fehm, Gunter Meister, Christoph A. Klein. Her2 and progesterone signaling cooperate for early dissemination of breast cancer cells. **Nature**. 2016 Dec 14. Doi: 10.1038/nature20785. PMID: 27974799.
6. Linde N, Fluegen G, Aguirre-Ghiso JA. The Relationship Between Dormant Cancer Cells and Their Microenvironment. **Adv Cancer Res**. 2016 ;132:45-71. doi: 10.1016/bs.acr.2016.07.002. Epub 2016 Aug 25. PMID:27613129.

Manuscripts Accepted:

1. Nina Linde, Arthur Mortha, Eduardo Farias, Maria Soledad Sosa, Kathryn Harper, Ethan Tardio, Miriam Merad, and Julio A. Aguirre-Ghiso. Macrophages orchestrate early dissemination of HER2+ cancer cells.– *Manuscript accepted in Nature Communications*.

Oral presentations during the funding period:

- 2015: Speaker, TMEN Steering Committee Meeting, 21-23 January 2015. Seattle, WA
- 2015: Keynote Speaker – Porto Cancer and Stem Cells meeting, IPATIMUP, Porto, Portugal.
- 2015: Invites speaker – FASEB SRC on TGF β signaling, Snowmass CO, USA.
- 2015: Invited Speaker – University of Colorado Cancer Center, Denver CO, USA.
- 2015: Invited Speaker – University of Kentucky, Lexington, KY, USA.
- 2015: Speaker – Samuel Waxman Cancer Research Foundation Annual Symposium, New York, NY, USA.
- 2015: Invited Speaker - CNIO Metastasis Meeting, Madrid, Spain.
- 2015: Invited Speaker - Abbvie – Chicago, IL – USA
- 2015: Invited Speaker - AACR Tumor Metastasis Meeting, Austin TX – USA
- 2016: Invited Speaker - TMEN Steering Committee Meeting. OSHU, Portland OR.

- 2016: Invited Speaker – Distinguished Seminar Series – Wistar Institute, Philadelphia, PA.
- 2016: Invited Speaker – Distinguished Seminar Series – Albert Einstein College of Medicine, Bronx, NY.
- 2016: Invited Speaker – Biological Sciences Department Seminar Series, University of Southern California, Los Angeles, CA
- 2016: Invited Speaker – Mayo Clinic Research Fellow Association Seminar Program, Mayo Clinic, Jacksonville, FL.
- 2016: Invited Speaker – Perlmutter Cancer Center Research Seminar Series, Laura and Isaac Perlmutter Cancer Center at NYU Langone, NY.
- 2016: Invited Speaker - FASEB 3rd International Conference on Retinoids. West Palm Beach, FL.
- 2016: Invited Speaker – Cancer Center Seminar Series, University of Florida, Gainesville, FL.
- 2016: Invited Keynote Speaker - XXXI International Congress of the International Academy of Pathology and 28th Congress of the European Society of Pathology, Cologne, Germany.
- 2016: Invited Speaker – Cancer Center Seminar Series, University of Dusseldorf Dusseldorf, Germany.
- 2016: Invited Speaker – Experimental Medicine and Therapy Research University of Regensburg, Fraunhofer Project Group, Personalized Tumor Therapy, ITEM Regensburg Germany.
- 2016: Invited Plenary Speaker – 2nd Novel Concepts in the Biology and Treatment of Metastatic Cancer Conference Fusion Conferences, Paphos, Cyprus.
- 2016: Invited Speaker – Immunology Research Seminars, Mayo Clinic, Rochester, MN.
- 2016: Invited Speaker – Metastatic Cancer Task Force – Department of Defense Murtha Cancer Center at Walter Reed Bethesda, MD.
- 2017: Invited Speaker – MD Anderson Cancer Center, TX.
- 2017: Selected work Speaker , AACR Annual meeting, Washington DC
- 2017: Invited Speaker – MD Abderson Cancer Center – NCI Workshop on Systems Biology of Metastasis
- 2017: Invited Speaker – Annual Samuwl Waxman Cancer Research Foundation Symposium.
- 2017: Invited Speaker – MPM – Astellas Scientific Colloquium, Boston, MA
- 2017: Invited Speaker – Van Andel Research Institute Cancer Research Seminar Series, Grand Rapids, MI
- 2017: Invited Speaker – department of Cell Biology Seminar Series, Johns Hopkins University, Baltimore MD
- 2017: Invited Speaker - AACR Special Conference on Tumor Immunology and Immunotherapy
- 2017: Invited Speaker - Cell Press LabLinks 2017 entitled "Cancer Metastasis".
- 2017: Invited Speaker – Disseminated Cacer Cell Network Retreat, University of Dusseldorf, Dusseldorf, Germany.

Posters

NA

Awards

NA

CONCLUSION

Summary of findings:

Progress has been highly significant as we demonstrated that early pre-invasive breast cancer lesions contain P-TMEM structures and provided evidence for the role of P-TMEM associated macrophages in early dissemination and subsequent metastasis formation. We showed that this was driven by the induction of EMT due to macrophage-derived Wnt. We further revealed the signaling pathway recruiting macrophages and characterized P-TMEM associated macrophages in detail. This was complemented by the first video documentation of intravasation and local invasion in early lesions performed in collaboration with the partnering PI, Dr. John Condeelis. The productivity is highlighted by the publication of this work in three papers, two in Nature and one accepted in Nature Communications.

We also found that there was a significant association of intra-epithelial macrophages with low E-Cadherin levels in DCIS patients. We further demonstrated that macrophages as S-TMEM components are able to induce DTC proliferation but this depended on the macrophage subtype: while lung resident macrophages drive proliferation, BM resident macrophages rather inhibit proliferation and possibly even induce dormancy.

Additionally, we successfully isolated early and late cancer cells both from the primary and the secondary target organ site and submitted these samples for RNAseq analysis. This will allow us to investigate the expression profile of early and late cancer cells and specifically how these might act to establish an S-TMEM. We also used an evidence-based approach and identified TWIST as a potential regulator of early DTC dormancy and that the loss of E-cadherin expression is also linked to early DTC dormancy. Overall, our findings support our hypothesis that early dissemination contributes to metastasis but also to a dormant DTC population that may be reactivated from dormancy by macrophages in the lung while DTCs in the bone might escape a reactivation from dormancy through BM resident macrophages. Our identification of the origins of DTCs may help to understand their biology and how to suppress dissemination and avoid metastasis formation.

Significance:

Our work is revealing an unexpected contribution of early DTCs to dormancy and metastasis in cooperation with macrophages. Our work challenges the current model of linear metastasis formation and argues that a better understanding of disseminated disease is required and might allow for novel therapies that target dormant DTCs and prevent metastasis formation. The findings that macrophages may also fuel early DTC metastasis suggests that in addition to therapies targeting the cancer cells we may be able to use therapies that also modulate macrophage subtypes at TMEM sites to block progression. This has been tested in principle by the Condeelis lab^{7,8}. Our work is highly relevant as it provides insight into early dissemination during stages that have commonly been considered as pre-invasive and thus harmless. Yet, recent studies have caused great confusion on how to treat women with stage 0 breast cancer. A recent study published in JAMA Oncology adds to this confusion as it shows that while breast cancer deaths from DCIS metastasis are ~3% of the whole population, 50% (thousands of women each year) die of metastasis in the absence of an invasive breast cancer recurrence and the choice of therapy did not affect survival. This suggests that DTCs remain untouched by therapies and that a subpopulation of women with stage 0 breast cancer carry early disseminated cancer cells that can have deadly consequences. We therefore need better tools to identify those stage 0 patients at high risk of developing late metastatic relapses without overtreating the majority of women who have harmless variants of DCIS. We believe that our work provides significant progress towards identifying markers to differentiate high risk and low risk cases of early breast cancer stages.

The analysis of the expression profiles of early and late cancer cells as well as the markers found in the DCIS clinical study led by Tanja Fehm from the University Clinic Dusseldorf, Germany, will also contribute to a better understanding of the metastatic disease.

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Macrophages orchestrate breast cancer early dissemination and metastasis

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Abstract

Cancer cell dissemination during very early stages of breast cancer proceeds through poorly understood mechanisms. Here we show that a previously described HER2⁺/P-p38^{lo}/TWIST^{hi}/E-cadherin^{lo} early-evolved cancer cell subpopulation requires macrophages for early dissemination. Depletion of macrophages only during premalignant stages reduces early dissemination but also metastatic burden at end-stages of cancer progression. In premalignant lesions, CCL2 produced by early HER2⁺ cancer cells and myeloid cells attracts CD206⁺/Tie2⁺ macrophages. In macrophages CCL2 induces Wnt-1 upregulation that in turn downregulates E-cadherin junctions in HER2⁺ early cancer cells. Premalignant lesions also show macrophage-containing tumor microenvironments of metastasis (TMEM) structures where intravasation can occur. A pilot analysis shows that intra-epithelial macrophages and loss of E-cadherin junctions is found in human ductal carcinoma *in situ* (DCIS). We reveal that in mouse models macrophages play a causal role in early dissemination affecting long-term metastasis development much later in cancer progression.

Introduction

The paradigm of cancer metastasis states that dissemination and metastasis occur when advanced aggressive tumors acquire invasive mechanisms. The finding that dissemination does not only occur from evolutionary late stage invasive tumors has challenged the uniqueness of this model¹. Large cohort patient studies²⁻⁵ and studies with spontaneous mouse tumor models⁶ showed that dissemination also occurs during early stages of cancer when lesions are diagnosed by light microscopy as pre-malignant or pre-invasive. In addition, cancer of unknown primary is a relatively frequent event in solid cancers where metastases develop without the presence of an obvious primary tumor mass that evolved to become invasive⁷.

The “early dissemination” definition was refined by Husemann et al.⁶ when they showed that early disseminated cancer cells (DCCs) originate at times when lesions are only defined *in situ* by light microscopy (e.g. ductal carcinoma *in situ* (DCIS) in humans and mammary intraepithelial neoplasia in mice) but dissemination occurs and early DCCs show few genetic aberrations. Following previous work⁶, we found that in MMTV-HER2 early lesions there is a sub-population of HER2⁺/P-p38^{lo}/P-ATF2^{lo}/TWIST^{hi}/E-cadherin^{lo} disseminating cancer cells that reach distant organs and initiate metastasis^{8,9}. Our studies revealed that HER2+ early cancer cells deregulate mechanisms of motility and invasion activated during mammary tissue branching morphogenesis^{8,9}. The remarkable finding was that early DCCs are endowed with latent metastasis initiating capacity^{8,9}. Women treated for DCIS can develop metastases without ever developing any subsequent local invasive breast cancer¹⁰⁻¹⁴. This might indicate that, albeit at low frequency, early DCCs can unpredictably form metastases in patients. Early

dissemination is not a rarity of breast cancer models (MMTV-HER2 and -PyMT models^{6,8,9}), as it also occurs in spontaneous mouse models of melanoma¹⁵ and pancreatic cancer¹⁶.

The mechanistic analysis of early dissemination has been primarily early cancer cell-centric^{8,9}. Since early DCCs displayed fewer genetic alterations than the late DCC counterparts^{4,6,8}, and the mechanism of dissemination resembled steps of mammary morphogenesis^{8,9}, we hypothesized that early dissemination might be driven by micro-environmental mechanisms that control epithelial cell motility and invasion during mammary tissue development^{17,18}.

The mammary epithelium forms post-natally during adolescence in a process called branching morphogenesis where rapidly dividing epithelial cells elongate the terminal end bud into the fat pad and bifurcate into the ductal tree. Macrophages are key regulators of branching morphogenesis during mammary gland development^{19,20}, arguing that normal mammary epithelial cells cooperate with these innate immune cells for invasive processes. These data led to the discovery of macrophages as powerful drivers of intravasation from invasive breast cancer tumors *via* the establishment of tumor microenvironments of metastasis (TMEM)²¹. This follows a streaming process where breast cancer cells recruit macrophages through colony stimulating factor 1 (CSF1) production and then cancer cell motility is stimulated *via* macrophage-derived epidermal growth factor (EGF)²². Additionally, macrophages can induce an epithelial to mesenchymal transition (EMT) in malignant cells^{23,24}. Elegant studies by Pollard and colleagues have also shown that macrophages play key roles in the maintenance of

lung metastasis^{25,26}. However, the role of macrophages in the process of dissemination during evolutionary early stages of breast cancer progression remained unexplored.

Here we show that the branching morphogenesis program is altered by oncogenes early in cancer development. CD206^{hi} macrophages in the mammary tissue are attracted by early cancer cells from the stroma into the epithelial layer of lesions defined as mammary intraepithelial neoplasia in mice (similar to DCIS in humans)²⁷. In the MMTV-HER2 model this process depends on HER2-NFkB-mediated induction of CCL2. Our data suggests that intra-epithelial macrophages respond to CCL2, which in turn can stimulate macrophages to produce Wnt-1, leading to disruption of E-cadherin junctions between early cancer cells. Before tumors form, these events result in early dissemination microenvironments that propel active intravasation and dissemination to the lung, which was efficiently blocked by macrophage depletion. Transient depletion of macrophages in mice before the formation of invasive tumors reduced lung metastatic burden later in mice life. Our results suggest a previously unrecognized role for local attraction of macrophages from the stroma into the ductal epithelium to favor dissemination of cancer cells much earlier than growth is induced by the oncogene. Our work also reveals a role for early DCCs in supporting late metastasis development, which is agreement with our recent studies supporting that ~79% of metastases in the MMTV-HER2 model descend from early DCCs.

RESULTS

MACROPHAGES INFILTRATE HER2+ EARLY LESIONS

We asked whether the HER2 oncogene might attract macrophages to orchestrate early dissemination. We used MMTV-HER2 mice as a murine model of breast cancer since these show slow progression from early lesions such as hyperplasia and mammary intra-epithelial neoplasia (**Fig.1A,B**), the latter a similar lesion to DCIS²⁷, to invasive tumors (**Fig.1C**). We stained MMTV-HER2 mammary gland sections for the murine macrophage marker F4/80 before we could detect any signs of invasive tumor masses in serial sections of mammary tissue or even enhanced proliferation in HER2+ early lesions²⁸. Macrophages were located to the stroma outside of mammary ducts in healthy FVB *wild type* animals (**Fig.1D**). This was also true in young 14wk old MMTV-HER2 mice (**Fig.1E**). However, when MMTV-HER2 mice progressed over time but still showed no signs of tumor masses or enhanced proliferation²⁸, macrophages were often localized inside the luminal epithelial layer of early lesions as demonstrated by co-staining of F4/80 and cytokeratin 8/18 (**Fig. 1F**). We hypothesized that as macrophages enter the early lesions, they might disrupt the architecture of the duct. Close inspection of sections co-stained for α -smooth muscle actin and F4/80 showed that the myoepithelial cell layer was frequently disrupted at sites where macrophages were in immediate contact with the duct (**Fig. 1G-I**). Quantification of the abundance of intra-epithelial macrophages confirmed that the incidence of ducts with intra-epithelial macrophages correlated with HER2 upregulation and disease progression (**Fig. 1J**).

MACROPHAGES DISMANTLE E-CADHERIN JUNCTIONS IN EARLY LESIONS

We hypothesized that HER2 might aberrantly activate a mechanism of invasion and motility involving macrophages in early lesions. We found that intra-epithelial macrophages were associated with a strong local downregulation of E-cadherin junctions *in vivo* in early lesion cells located directly adjacent to macrophages (1-2 cell diameter away) (**Fig.2A-C**). This was paralleled by a general downregulation of E-Cadherin mRNA in early lesions compared to wild type glands (**Fig.2D**), which had been reported previously⁹. Additionally, β -Catenin levels (blue signal **Fig.2E-G**) were increased in early lesions containing intra-epithelial macrophages as measured by dual color immunohistochemistry (IHC) (**Fig.2E-G**). A loss of E-Cadherin and translocation of β -Catenin to the nucleus could also be induced *in vitro* when Raw264.7 macrophages were added to Comma-1D healthy mammary epithelial cell monolayers used as readout for epithelial junction formation (**Fig.2H-J and 2,M,N,P**). The loss of E-cadherin junctions in epithelial cells adjacent to macrophages suggested that macrophages might produce cues that stimulate a loss of E-cadherin junctions as observed during the epithelium to mesenchyme transition (EMT). Macrophages can produce Wnt ligands²⁹⁻³¹ which are potent inducers of an EMT. We therefore tested the response of Raw264.7 macrophages or primary mammary tissue macrophages isolated from early lesions to conditioned media from healthy epithelial cells or from HER2+ early cancer cells. Only conditioned media derived from HER2+ cells induced an upregulation of Wnt-1 (**Fig.2K, L**); no changes were detected for Wnt3a upon treatment with the HER2+ cells conditioned media and Wnt5a and Wnt7 were not detectable in either mammary tissue macrophages or RAW264.7 cells (**Supplementary Figure 1G**). Our published data

also showed that Wnt-1 is not produced by the early lesions^{8,9}. Comma-1D cells also do not produce Wnt-1³², arguing that macrophages are the main source of Wnt-1. However, HER2+ early cancer cells do produce other Wnt ligands^{8,9} that could further enhance a Wnt signaling pathway. The loss of E-Cadherin junctions in Comma-1D cells induced by Raw264.7 macrophages was reversed by addition of DKK1, an inhibitor of canonical Wnt signaling, to the co-cultures (**Fig.2M-P**). Further, Comma-1D cells exposed to conditioned media from Raw264.7 cells immuno-depleted *via* precipitation from Wnt-1 with a specific antibody (**Supplementary Fig.1A-B**) were able to restore, E-cadherin junctions, when compared to IgG depleted conditioned media that displayed marginal E-cadherin junction formation (**Supplementary-Fig.1A-B**). Our data support that downregulation of *Cdh1* mRNA and junctions and β -catenin nuclear translocation in early lesion cells results from HER2-dependent attraction of Wnt-1 secreting macrophages from the stroma into the early lesions. Because, HER2+ early cancer cells also produce other Wnt ligands we further conclude that the downregulation of E-cadherin junctions may be due to the combined effect of early cancer cell- and macrophage-derived Wnt ligands.

Macrophages that produce Wnt ligands³¹ are known to associate with tumor cells that interact with the TMEM structures. A TMEM structure is composed of a macrophage, a MENA^{high} tumor cell and endothelial cells and serve as the portal for intravasation^{33,34}. The presence of macrophages in early lesions prompted us to test if TMEM are formed in these HER2 early lesions as found in the PyMT model early lesions²¹. Using a clinically validated test³⁴ it was shown that early cancer lesions in the MMTV-HER2 and MMTV-PyMT²¹ models were efficiently forming TMEM

(**Supplementary Fig1C-D**). Our data (**Supplementary Figure 1C-D**) reveals that the HER2+ model also shows formation of TMEM structures which can operate as portals for intravasation^{33,34}. These TMEM structures occur in approximately 10% of ducts at 17-week old MMTV-HER2 animals (**Supplementary Figure 1E**). This frequency is in range with the frequency of intraepithelial macrophages in premalignant lesions in **Fig1J**. Importantly, compared to *wild type* mammary tissue, early lesions in the MMTV-PyMT model also showed a significant ($p \leq 0.0001$) down-regulation of E-cadherin (**Supplementary Fig2A**), arguing that PyMT-initiated signaling might converge on similar signals as HER2 to attract macrophages from the stroma into the early lesions and downregulate E-cadherin junctions. As reported²¹, normal mammary tissue in FvB mice was negative for TMEM (**Supplementary Figure 1F**).

MACROPHAGE DEPLETION PREVENTS EARLY DISSEMINATION

We next tested whether macrophage mobilization into early lesions where they induce an EMT in early cancer cells leads to early dissemination. CSF1R is expressed by most tissue resident macrophages and is required for macrophages survival in tissues³⁵. Thus, we injected MMTV-HER2 mice during early lesions with a blocking antibody to CSF-1 receptor (CSF1R) to eliminate macrophages from early lesions or as controls with PBS or an isotype matched IgG (**Fig.3A and Supplementary Fig 2B**). CSF1R blockade led to the depletion of tissue resident $CD11b^+/F4/80^+/Gr1^-$ macrophages compared to PBS and IgG treated animals (**Supplementary Fig.2B-C**). Importantly, IgG control did not alter the relative or total levels of $F4/80^+CD11b^+$ macrophages, $CD11b^+F4/80^{NEG}$ monocytes or $F4/80^+CD206^{HI}$ or $+CD206^{LO}$ macrophages. Dendritic cell and neutrophil numbers and percentages from the $CD45^+$

gate were also not affected by the IgG (**Supplementary Fig.2C**). CSF1R-blocking antibody treatment did deplete significantly ($p<0.05$) the F4/80+CD206^{HI} macrophage population and monocytes while neutrophils and dendritic cells remain unchanged (**Supplementary Fig.2C**). Quantification of immunofluorescence staining images for F4/80 in HER2+ early lesions (**Supplementary Fig2E**) confirmed a significant reduction in the number of intra-epithelial macrophages when CSF1R was blocked compared to all controls (**Supplementary Fig.2B, C and E; $p<0.05$**). We confirmed that at the end of the experiment, no tumor masses had formed, by inspecting whole mounts of mammary glands (**Supplementary Fig.2G,H**) and hematoxylin-eosin (H&E) staining of serially sectioned mammary tissue (**Fig.3B,C**). Macrophage depletion was accompanied by a significant reduction in the number of hyperplastic ducts (**Fig 3B-G and Supplementary Fig.2F; $p<0.05$**) and a tissue wide upregulation of E-Cadherin mRNA (**Fig.3D**) and E-Cadherin-based junctions compared to PBS (**Fig.3E-G**) or IgG controls (**Supplementary Fig.2D**). Overall, we conclude that macrophages contribute to the loss of E-cadherin mRNA and junctions and disrupted mammary tissue architecture in early lesions.

The above changes correlated with the finding that CSF1R blockade significantly ($p=0.019$) reduced the numbers of early circulating cancer cells (**Fig.3H**). Accordingly, CSF1R blockade also reduced early-disseminated cancer cell (eDCC) burden in target organs as measured by the detection of the transgene surface HER2 expressing early DCCs in the lungs (**Fig.3I-K**). To rule out that HER2+ cells might be macrophages that engulfed early cancer cells in the mammary tissue and migrated to the lung, we tested the fraction of cells in the lungs in MMTV-HER2 animals that might be double positive

for HER2 and macrophage markers (F4/80) (**Supplementary Fig.3A**). Only ~ 2 cells per field of view (FOV) were HER2/F4/80 double positive vs. a median of 46 DCCs/FOV were HER2+/F4/80-. This results in a frequency of 0.04 or 4% of all HER2+ cells being double positive. This argues that only 4% of HER2+ cells could be confused for a macrophage engulfing HER2+ cells in the lung or traveling to the lung with the engulfed cells. We conclude that macrophages play a critical role in the ability of early cancer cells to acquire an invasive and disseminating phenotype.

EARLY DISSEMINATION MACROPHAGES FUEL LATE METASTASIS

We next tested whether macrophage-regulated early dissemination contributed to metastasis formation. To this end we blocked CSF1R only during early asymptomatic stages of cancer, starting at age week 18, and stopped as soon as tumors became palpable (size <3mm in diameter). We then waited until tumors reached 1 cm in diameter (4-6 weeks later) and quantified solitary DCCs and metastatic lesions in lungs (**Fig.4A**). We found that the time to tumor detection was slightly delayed when macrophages were depleted during asymptomatic pre-malignant stages (**Fig.4B**). However, once palpable tumors had formed, the progression to overt tumors was not affected (**Fig.4C**). Additionally, overt tumors showed no difference in macrophage content (**Fig.4D-F**) and vascularization (**Fig.4G-I**) at the end of the experiment between control and anti-CSF1R treated mice. This suggests that there is no impact on overt tumor growth in late tumors when macrophages were depleted during early stages of cancer progression. Additionally, flow analysis of lungs revealed that neither alveolar macrophage nor CD11b+/Gr1+ monocyte content was affected (**Supplementary**

Fig.3B-F) by the same treatment arguing against a non-specific depletion of macrophages. However, CSF1R blockade during early stages significantly ($p=0.028$) decreased solitary DCC burden in lungs (**Fig.4J**). CSF1R blockade during early stages also caused a statistically significant ($p=0.039$) decrease in the number of metachronous metastases per mouse (**Fig.4K-L**), which were defined as the number of metastatic lesions bigger than three cells (**Fig.4K-L**). Previous work⁹, which was confirmed here, showed that DCC clusters >5 cells tend to be positive for proliferation markers (P-Rb and P-Histone-H3) arguing that they are growing micrometastasis. This inhibitory effect of DCC burden and metastasis was detected even after macrophage depletion had been stopped in average for one month and animals had carried fast-growing tumors. We conclude that macrophages aid early dissemination of HER2+ early cancer cells, allowing for the early DCCs to reach target organs and form metastasis.

PROFILE OF MYELOID CELLS IN HER2+ EARLY LESIONS

We next performed an unbiased profiling of macrophages and other immune cells present in *wild type* and early lesions using time of flight mass cytometry (CyTOF) (**Fig.5A-C**) and conventional fluorescence-activated cell sorting (FACS) (**Supplementary Fig.4A-G**). Our data in the MMTV-Her2 model parallel the differences between wt mammary gland and tumor associated macrophages described previously in the MMTV-PyMT model³⁶. Our CyTOF analysis³⁷ showed that frequency and expression patterns of different myelo-monocytic cells was similar between wild type glands and early lesions, but differed from overt tumors (**Fig.5A and Supplementary Fig.4A-B**). Monocytes were identified based on their high Ly6C levels (**Fig.5B**) and their

percentage relative to CD45+ cells increased in early lesions compared to wt mammary tissue but this was not due to increase in numbers (**Fig.5B, 5D and Supplementary Fig4F**). The remaining LY6C^{negative} macrophages were distinguished based on CD206 expression (CD206^{HI} and CD206^{LO}). CD206^{LO} macrophages slightly increased in early lesions compared to wild type mammary tissue as detected by CyTOF and multicolor FACS (**Fig.5C, 5E**); CD206^{HI} macrophages were more frequent in wt and early lesions and did not change between wt and early lesions but decreased in frequency in overt tumors (**Fig5C, 5F and Supplementary Fig4E**). The changes in frequency for CD206^{HI} and CD206^{LO}, were not due to an increase in total macrophage numbers (**Supplementary Fig4E**). IdU incorporation analysis revealed that CD206^{LO} macrophages were more proliferative starting at week 14 throughout to over tumor stages, while CD206^{HI} macrophages remain non-proliferative (**Fig 5J**). In early lesions CD206^{HI} macrophages expressed slightly higher levels of CD11b than CD206^{LO} macrophages (**Supplementary Fig.4C**). Tie2 levels were significantly ($p<0.05$) different between CD206^{HI} and CD206^{LO} macrophages in either CyTOF or conventional FACS measurements (**Fig5I and Supplementary 4G**). We observed no changes in other lymphoid or myeloid cells (**Supplementary Fig4F**). Overt tumors, as expected, showed changes in almost all populations (**Supplementary Fig4F**). We next performed *in situ* staining to define the phenotype and location of intra-epithelial macrophages in early lesions, which cannot be resolved by FACS (**Fig.5K-M**). We found that stromal and intra-epithelial macrophages in both wild type glands and early lesions displayed higher CD206 mean fluorescence intensity than that detected in macrophages in overt tumors (**Fig.5N**). Our data suggest that circulation derived monocytes do not contribute to the

CD206 macrophage pool at early lesions. However, the IF analysis show that CD206^{hi} macrophages are the macrophage subset found within the stroma and intra-lesion compartments in early lesions but that intra-lesion macrophages express the highest CD206 levels, suggesting a local translocation (**Figure 5K-N**).

PREMALIGNANT CELLS ATTRACT MACROPHAGES VIA CCL2

We next focused on what signals might HER2 upregulation induce to attract local macrophages from the stroma into the early lesions. In invasive breast cancer models, HER2 signaling activates NFκB, which transcriptionally induces CCL2, a potent macrophage chemotactic factor³⁸. We found that the p65 subunit of NFκB subunit was phosphorylated in mammospheres derived from HER2+ early cancer cells, and Lapatinib, a HER2 and EGFR inhibitor, inhibited its phosphorylation (**Fig.6A**). We then isolated RNA from mammospheres derived from either FVB wild type or MMTV-HER2 early lesions as described⁹ and performed quantitative real-time PCR analysis for cytokine mRNAs. We found that already at these early stages of progression, HER2+ cancer cells upregulated expression of *Ccl2* but not *Csf2*, *Csf1*, *Il1β*, and *Il6* (**Fig. 6B, Supplementary Fig.5A,B**). *Csf2* mRNA was detectable in wt and early lesion-derived spheres but the expression was not different between these groups and could not be confirmed by IF (**Supplementary Fig.5C,E**), while overt tumors showed detectable CSF2 arguing that early lesions do not express CSF2 (**Supplementary Fig5F**). Upregulation of CCL2 around HER2+ early cancer cells was also observed at the protein levels as confirmed by quantifying soluble CCL2 in the conditioned media of the early cancer cells by ELISA and by IF (**Fig.6C,D, Supplementary Fig.5G-H**).

Quantification of the increase in CCL2 IF signal (**Supplementary Fig5H**) in the HER2+ cells vs. wt epithelium in **Fig6C** revealed a difference that was confirmed by ELISA and qPCR from isolated mammospheres from the same primary tissues. CCR2+/CCL2+ cells could be found close to CCL2+ early cancer cells that showed lower signal for both CCR2 and CCL2; these signals were undetectable in wt tissues (**Fig.6C insert shows CCR2 and Supplementary Figure 5I**). Q-RT-PCR of RNA isolated from FACS-sorted monocytes, macrophages, neutrophils and HER2+ cancer cells during early stages of progression confirmed the CCL2 production by HER2+ early lesions (**Supplementary Fig5J**), as detected by q-RT-PCR, IF and ELISA (**Fig6.B-D**). HER2+ early lesions produce lower levels of CCL2 mRNA than monocytes and macrophages, suggesting multiple sources of CCL2. Organoids produced by early MMTV-HER2 cancer cells displayed a reduction in secreted and peri-organoid CCL2 production upon inhibition of HER2 or NFkB signaling with specific inhibitors ³⁹ as detected by IF and ELISA (**Fig.6E-G and Supplementary Fig5G and K**). To confirm that CCL2 signaling was necessary and sufficient for HER2-dependent macrophage attraction, HER2+ early cancer cells were grown as threedimensional (3D) acinar structures *in vitro* for 5 days. Cultures were then treated with Lapatinib, an IKK β inhibitor ³⁹ or an inhibitor of the CCL2 receptor CCR2 (RS504393) and macrophages isolated from mammary glands of MMTV-HER2 mice were added to the cultures. After 24 hours of co-culture, macrophages were associated with ~50% of all organoid structures in control samples (**Fig.6H**). In contrast, co-cultures treated with the inhibitors all showed significant (p<0.04) reduction in macrophage association (**Fig.6I,J**). When we tested whether CCL2 was responsible for Wnt1 production by macrophages, we found that Raw264.7 cells treated with

recombinant CCL2 at doses known to stimulate chemotaxis⁴⁰, produced more Wnt1 than control treated cells (**Supplementary Fig5L**). We next treated 20 week old MMTV-HER2 mice carrying only early lesions (no palpable or overt tumors) for 2 weeks with a CCR2 inhibitor (**Fig.6K,L**). We found that the number of intra-epithelial macrophages was significantly ($p=0.004$) reduced (~40%) when mice were treated systemically (**Fig.6M**). However, systemic CCR2 inhibition did not reduce the number of eCCCs (**Fig.6N**) compared to the treatment that blocked the CSF1R which reduced intra-epithelial macrophages by ~77% (**Fig3H**). It is possible that the CCR2 inhibitor is less potent in blocking macrophage translocation than the anti-CSF1R antibody as evidenced by the stronger inhibition in macrophage translocation into the mammary tissues. When the CCR2 inhibitor was administered locally into the fat pad to avoid more widespread systemic effects (**Fig.6N**), intra-epithelial macrophage content was reduced compared to contra-lateral control treated glands (**Fig.6O**). We conclude that HER2 signaling in cancer cells from early lesions activates NFkB to induce CCL2, but that other myeloid cells also produce significant CCL2 mRNA. We further conclude that CCL2 may be a signal that stimulates macrophages to produce Wnt-1 as shown in **Fig2K-L**.

INTRA-EPITHELIAL MACROPHAGE IN PATIENT DCIS LESIONS

Published data showed that more than 10% of patients with DCIS had detectable DCCs in their bone marrow (BM), but no histologic markers, which include invasive fronts and receptor status were indicative of the presence of DCCs³. Macrophages are detected in the stroma of normal breast tissue⁴¹. To test whether macrophages could

also infiltrate early lesions in humans, we undertook a pilot analysis of a small number of patients samples and compared macrophages in healthy human breast tissue vs. tissue from DCIS lesions as a model of early stage breast cancer lesions. Macrophages were identified as CD68⁺/CD45⁺ and cytokeratin8/18 negative cells (**Supplementary Fig.6A, B**). As reported⁴¹, in breast tissue from healthy donors (n=7), CD68⁺/CD45⁺/CK8/18⁻ macrophages were localized in the stroma in the vicinity of mammary ducts but remained outside of the ducts, which were delimited by an intact myoepithelial layer of cells (**Fig.7A**). In contrast, even in DCIS lesions that displayed an apparently intact myoepithelial layer, there was a statistically significant (p=0.0006) increase in the frequency of macrophages found inside the aberrant ductal epithelial structure in between cancer cells (n=10) (**Fig.7B,C**). The intra-epithelial CD68⁺/CD45⁺/CK8/18⁻/E-cadherin⁻ macrophages were commonly associated with cancer cells (CD68⁻/CD45⁻) that showed reduced E-Cadherin levels in human DCIS samples (N=12) as measured by quantitative image analysis (**Fig.7D-F**). Additionally, within the same patient, individual lesions with high macrophage numbers had lower E-Cadherin levels (**Supplementary Fig.6C-E**). Additional breakdown in grade subgroups showed that vs. healthy tissue there was no increase in intra-lesion macrophages when compared with low-grade DCIS lesions (**Supplementary Fig.6F**). However, high-grade lesions showed a significant (p<0.04) increase in intra-lesion macrophages when compared to healthy and low-grade tissue (**Supplementary Fig.6F**). Quantification of intra-lesion macrophages in HER2- or HER2+ DCIS lesions revealed no differences (**Supplementary Fig.6G**). This work is limited by the low number of patients. However, the pilot data suggests that other oncogenic signals associated with a high grade

histological subtype in humans could also result in macrophage attraction into the lesions. These may be signals as those propagated by the PyMT oncogene as macrophages in TMEM structures were detected in early MMTV-PyMT lesions (Supplementary Fig1C-E and 2A).

Discussion

Our published work has shown that oncogene-driven early dissemination is due to the co-option by oncogenic pathways of mechanisms of motility and invasion activated during mammary branching morphogenesis^{8,9}. This included the activation of cell intrinsic pathways that through an EMT-like program propelled early dissemination and metastasis^{8,9}. Importantly, early dissemination was documented in humans in breast and pancreatic cancer^{6,42}. Our data suggest that macrophages enter the epithelium of early lesions in mice where they create early dissemination microenvironments that in both the PyMT and HER2 models had all the components of TMEM^{33,34}. Based on our data we hypothesize that HER2+ early cancer cells attract macrophages locally and in response to the CCL2 signal these macrophages also produce CCL2, as macrophages from wt mammary glands expressed lower levels of CCL2. We further hypothesize that these IEM macrophages produce Wnt1 in response to CCL2 to dismantle epithelial E-Cadherin junctions. That the CCR2 inhibitor did not result in a decrease in eCCCs may be due to dosing limitations or to a stronger dependence of eCCC precursors on CSF1 than CCL2. Nevertheless, our in vitro 2D and 3D organoid culture data allows us to propose that, while early cancer cells produce several Wnt ligands^{8,9}, Wnt1 may be exclusively produced by macrophages. However, we acknowledge that a limitation of our study is the absence of *in vivo* validation of the Wnt-1 role in the context of early

dissemination. The dissemination-promoting function of macrophages was proven when we found that depletion of macrophages in early HER2+ lesions using anti-CSF1R antibodies reversed the loss of E-cadherin in HER2+ lesions as well as intravasation and dissemination to lungs. Interestingly, Wnt signaling is linked to branching morphogenesis^{43,44} and a subset of tumor-associated macrophages that drive invasive cancer dissemination through TMEM formation also secrete Wnt ligands³¹ while CCL2 production by colorectal cancer cells can also foster vascularization and intravasation as shown in more advanced tumors⁴⁵. The detection of TMEM in the PyMT and HER2 early lesions not only supports that these mechanisms are not HER2-exclusive, but also that these structures that orchestrate intravasation and have a clinical prognostic value³⁴ may be operational at earlier time points than expected from the invasive cancer paradigm.

We found that the myelo-monocytic landscape of HER2+ early lesions at the time of early dissemination resembled that of healthy mammary glands and paralleled the findings in the PyMT model³⁶. Early lesions contained both F4/80+/CD11b+/CD206^{hi}/Tie2^{hi} but also F4/80+/CD11b+/CD206^{lo}/Tie2^{lo} macrophages. In contrast, overt tumors predominantly contained CD11b^{int}/CD206^{lo}/Tie2^{hi} macrophages as described³⁶. *In situ* imaging supported that F4/80/CD206^{hi} macrophages may be more frequently intraepithelial but the total numbers and proportions in the mammary tissue do not differ from the CD206^{lo} population. That CD206^{hi} populations of macrophages were Tie2^{hi} suggest that they might function as a subset of macrophages in invasive breast cancer lesions that are gatekeepers of intravasation doorways²¹. Our results raise the possibility that when HER2 or other

oncogenic signals are activated in mammary epithelial cells, macrophages that translocate into the early lesions from the stroma have the inherent potential to aberrantly fuel epithelial cell motility as observed during mammary gland development^{19,20,46}. However, further scrutiny and lineage tracing experiments would be required to fully understand the origin of macrophages driving early dissemination.

We found that when macrophages were depleted during early stages but allowed to rebound during invasive stages, lung metastatic burden was reduced. This result indicates that early DCCs contribute to lung metastasis formation, either directly or indirectly. Surprisingly large tumors that persisted in mice for ~1.5 months were not able to compensate for the reduced macrophages and dissemination during early stages. This may be due to the lower dissemination capacity we observed in HER2+ overt tumors vs. early lesions⁸. Further, that the number of solitary DCCs, likely a mixture of DCCs accumulated since the early stages, was reduced by CSF1R blockade suggests that the reduced influx of DCCs to lungs during early stages was not replaced by DCCs arriving during the time of tumor detection to euthanasia when tumors were large. This is in agreement with our recent phylogenetic data that a fraction of metastasis detected late in MMTV-HER2 animals were indeed derived from early DCCs and not from the overt primary tumor⁸ (**Fig.8A** scenario 1). Additionally, early DCCs may cooperate with later arriving DCCs to form metastasis in patients that after DCIS treatment go on to develop invasive lesions or in patients that had DCIS but only were diagnosed later for invasive cancer (**Fig.8B** scenario 2). The low number of patients in our pilot analysis and the fact that there might be mouse to human differences in macrophage function limits the conclusions of our study on the human relevance of our findings.

Nevertheless, it encourages further testing of human samples to determine whether detection of macrophages and E-cadherin levels in DCIS lesions may help predict which patients have early dissemination.

Early dissemination may also impact the concept of the “pre-metastatic” niche^{48,49} where this microenvironment could be orchestrated by early DCCs in addition to bone marrow derived cells. Qian et al showed that in MMTV-PyMT invasive cancer models, VEGFR+/CCR2+/CXCR4-/Tie2- macrophages fuel metastasis in the lung²⁶. Our data show that macrophages rebound in tumors during overt growth stages. Still even after this rebound, metastasis were reduced if the anti-CSF1R-mediated depletion of macrophages was done during early stages. We propose that the VEGFR+/CCR2+/CXCR4-/Tie2- macrophage-mediated support of metastasis may not be able to compensate for the reduction in DCCs caused by macrophages depletion during early dissemination in primary sites. However, we cannot rule out the possibility that the anti-CSF1R mAbs affected the population described by Qian et al²⁶ in lungs. Thus, it is possible that during early stages, depletion of VEGFR+/CCR2+/CXCR4-/Tie2- macrophages also impacted early DCC lodging in lungs. This is an interesting possibility we will explore in future studies.

Overall, our studies suggest that oncogenes might turn on developmental programs of anoikis resistance²⁸, migration and invasion^{8,9} and macrophage translocation into the early lesions much earlier than anticipated. These programs then initiate early dissemination before propelling rapid tumor growth. Overall, in this study, we provide critical new insight into the understanding of the natural history of metastatic disease in mice and we demonstrate that early in cancer evolution macrophages and

early DCCs appear to play a seminal role in metastatic breast cancer that can manifest later in life of these mice.

Methods

Cells and cell culture

Raw264.7 cells were received from ATCC. Transfection with mCherry was done using mCherry lentiviral vectors and maintained in DMEM (Lonza) with 10% FBS and 1% Pen/Strep. Comma-1D cells were a generous gift from Daniel Medina and were maintained in DMEM-F12 medium containing 2%FBS, 1% Pen/Strep. For DKK1 stimulation, conditioned media was prepared from DKK1 expressing 293T cells which were a gift from S. Aaronson. For preparation of conditioned medium, protein concentration was determined using a Bradford assay cells cultured with serum free medium (DMEM + 1%P/S) for 24 hours and then concentrated using Vivaspinn 20 Centrifugal Concentrating tubes (Sartorius, VS2021) at 3000g up to 3 hours until desired concentration (10x) was reached. 0.5ug/ml DKK1 protein was used for stimulation. For co-culture experiments, Comma-1D cells were seeded on coverslips and after 12h, Raw-264.7-mCherry cells were added. Co-cultures were fixed in 2% formalin after 12h and then stained. All cell lines were routinely tested for mycoplasma. Raw-264.7-mCherry cells were treated with recombinant CCL2 (20 ng/ml, catalog number: 250-10, Peprotech) and 24 hours later the levels of WNT1 were detected by QPCR. Comma 1D cells were cultured with the conditioned medium from Raw-264.7-mCherry cells (24 hrs, diluted 1/40) and after 24 hours the percentage of cells forming

E-cadherin junctions were detected by IF. Raw-264.7-mCherry conditioned medium (CM) was incubated overnight with anti-WNT1 ab (ab15251, Abcam) or IgG control, then depleted from CM and then added to Comma 1D cells.

Mouse experiments

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Icahn School of Medicine at Mount Sinai, protocols 08-0366 and 2014-0190. Mice used were female FVB/N-Tg(MMTVneu)202Mul/J or FVB wild type mice and were purchased from Jackson laboratory and bred in-house. Animals were sacrificed using CO₂ at age 14, 20-22wk or when invasive tumors had reached a diameter of 1cm. For macrophage depletion, we administered 3mg of the CSF1R antibody clone ASF98 on day 1 and 1mg on day 7 and weekly thereafter by injection into the tail vein of 18wk old pre-malignant MMTV-Her2 mice. PBS was used as vehicle control. Treatment lasted 14 days or until tumors were first palpable (3mm diameter). ASF98 antibody was a generous gift of Dr. Miriam Merad. For CCR2 blockade, mice were either treated with 2mg/kg of RS504393 (Tocris Bioscience) or vehicle control (DMSO) daily by i.p. injections for 14 days or by injection of 1mg/kg RS504393 into the fat pad for 5d.

518 Mammary Gland Whole Mounts

519 Mammary glands were excised, spread on glass slides and fixed overnight in 4%
520 formalin at 4°C. Glands were then dried for 5min and fixed in Carnoy's fixative (60%
521 ethanol, 30% chloroform, 10% glacial acetic acid) for 2-4h at room temperature. Glands
522 were washed in 70% ethanol for 15min and in 50% ethanol twice for 15min each and
523 rinsed with distilled water for 5min. Staining with carmine alum solution occurred over
524 night (0.2% carmine, 0.5% aluminium potassium sulfate in water boiled for 20min and
525 then filtered). Glands were washed in 70% ethanol for 15min, in 96% ethanol and in
526 100% ethanol for 15min and in xylene for 15min and stored in methyl salicylate.

527

528 Microscopy

529 Formalin fixed and paraffin embedded samples were prepared and stained as described
530 ⁵⁰. For immunohistochemistry, VectaStain Elite ABC Rabbit IgG and Mouse IgG (PK-
531 6102) kits from Vector Laboratories were used for secondary antibodies. Secondary
532 antibodies were left for one hour at room temperature. DAB and Vector Blue substrate
533 kit (Vector Laboratories) were used for enzymatic substrate. Mounting was done using
534 VECTASHIELD mounting media (Vector Laboratories). For CD206 stainings,
535 cryosections were used. Tissue was fixed in 4% formalin overnight, incubated in 30%
536 sucrose/PBS overnight and sectioned into 6µm sections. Staining of cryosection was
537 done as described ⁵¹. Antibodies used were: CD68 (Sigma #MS397-PO 1:100), CD45
538 (Cell Signaling #13917P 1:100), F4/80 (abcam #ab6640 CIA:3-1 1:100), Iba-1 (Wako
539 #019-19741 1:200), Cytokeratin 8/18 (Progen #GP11 1:200), smooth muscle actin
540 (Sigma #A2547 clone IA4 1:200), CD206 (Biolegend #141073 clone 068C2 1:100),

CCL2 (Novus #NBP2-22115 clone 2D8 1:100), CCR2-647 (Biolegend #150603 clone SA203G11 1:50), E-Cadherin (Becton Dickinson #610181 cone 36/E 1:100), beta catenin (cell signaling #8480S clone D10A8 1:100), Her2 (abcam #ab2428 1:100), Endomucin (Santa Cruz #sc-65495 clone 7C7 1:100). For costaining of F4/80 and CD206 (both raised in rat) an A488-conjugated F4/80 antibody (Biolegend #123119 clone BM8 1:50) was used in combination with CD206 (Biolegend #141726 clone C068C2 1:100) in a sequential stain. Microscopic analysis was carried out with a Leica widefield microscope or with a Leica confocal microscope for 3D cultures. For quantification of immunofluorescence signal intensity with the Metamorph software, regions of interest were defined in original tiff files that had been taken under the same exposure time and settings and the mean signal intensity was measured. Because the use of a directly conjugated F4/80 antibody resulted in lower signal intensity, we used Iba1 as a macrophage marker ²¹ to identify macrophages for CD206 signal intensity measurement instead. For detection of micro- and macrometastasis we followed published protocols⁹. Briefly, the dormant non-proliferative lesions are mostly single cells of up to 10 cell clusters as defined by the absence of proliferation markers such as Ki67, P-Rb and P-ser10-Histone H3^{9,50,52,53}. In some studies, we found an upper limit of 20-cell clusters completely devoid of proliferation makers. For each study the analysis of proliferative vs. non-proliferative lesions is calibrated for a specific operator that is developing the study. In this paper, we found that 5-cell clusters were a good indication of a lower limit for what we call a proliferative micro-metastasis. This number was within range of our previous studies in the MMTV-Her2 model⁹.

Flow Cytometry

MMTV-HER2 mice were sacrificed using CO₂ at age 18-22wk (early pre-malignant cancer lesions) or when overt tumors had formed. Whole mammary glands or tumors were digested in Collagenase/BSA at 37°C for 30-45min. Red blood cell lysis buffer (Sigma) was used to remove blood cells. Cell suspensions were blocked with Fc-blocking reagent (eBioscience) and samples were surface stained in FACS buffer (PBS supplemented with 1% BSA and 2mM EDTA) for 20-30 min on ice using the following antibodies: CD45-PerCPy5.5 (Biolegend #103131 clone 30-F11 1:200), CD11b-PeCy7 (eBioscience #101215 clone M1/70 1:200), CD11c-PE (eBioscience #117307 clone N418 1:100), Gr1-AF700 (eBioscience #56-5931-80 clone RB68C5 1:200), Tie2-biotin (eBioscience #13-5987-82 clone TEK4 1:100), F4/80-biotin (Biolegend #123105 clone BM8 1:100), CD206-APC (Biolegend #141707 clone C068C2 1:100), VCAM-FITC (eBioscience #11-1061-82 clone 429 1:50). DAPI was used to label dead cells. Multiparameter analysis was performed on a Fortessa (BD) and analyzed with FlowJo software (Tree Star). DAPI+ cells and doublets were excluded from all analysis. To sort mammary tissue macrophages, whole mammary glands from 14wk, 18-22wk (early pre-malignant cancer lesions) mammary glands or from invasive tumors were digested in Collagenase/BSA at 37°C for 30-45min. Mononuclear cells were enriched in a Percoll gradient and then macrophages were sorted as viable CD45+/Gr1-/CD11b+/F4/80+ cells.

To study the myeloid and lymphoid mammary gland infiltrate, FVB/N WT, MMTV-HER2 18-22 weeks and MMTV-HER2 females with overt tumors were digested as above and incubated with a panel for myeloid markers: Tie2-PE (clone TEK4,

587 eBioscience # 12-5987-82 1:200); F4/80 APC (clone BM8, Biolegend #123115 1:200);
 588 CD11c PECy7 (clone N418, eBioscience #25-0114-82 1:200); MHCII AlexaFluor700
 589 (clone M5/114.15.2 eBioscience #56-5321-82 1:100); CD11b PerCPCy5.5 (clone M1/70
 590 eBioscience #45-0112-82 1:200); CD206 FITC (clone C068C2 Biolegend #141704
 591 1:200); CD106-biotin (clone 429, eBioscience #13-1061-82 1:200), Streptavidin APC
 592 eFluor780 (eBioscience #47-4317-82 1:500); Ly6G eFluor450 (clone 1A8, Biolegend
 593 #127612 1:200) and lymphoid markers (CD8 APC clone 53-67 eBioscience # 17-0081-
 594 82 1:200; CD4 PECy7 clone GK1.5 eBioscience #25-0041-82 1:200; CD3 PerCPCy5.5
 595 clone 145-2C11 eBioscience #45-0031-82 1:200; B220 FITC RA3-62B eBioscience
 596 #11-0452-82 1:200; CD19 eFluor450 clone eBio1A3 eBioscience #48-0193-82 1:200).
 597 Counting beads (40000 beads/ml) were added per tube (500 ul per tube) and 2000
 598 beads were acquired of every sample (Accucheck counting beads Life technologies,
 599 PCB100). Absolute numbers of CD45+ leukocytes were quantified as follows:

$$\frac{\# \text{ cells}}{\# \text{ cells acquired}} \times \frac{\# \text{ beads}}{1 \text{ ml}} \times \frac{0.5 \text{ ml}}{\text{tube}} = \# \text{ cells/tube}$$

601 Percentages obtained in flow cytometry among CD45+ cells (calculated as mentioned
 602 before) were used to obtain absolute cell counts of every population.

603

604 **Sorting of myeloid and CD45^{NEG} Her2+ populations**

605 Mammary glands from MMTV-Her2 females between 18-22 wks old were digested as
 606 mentioned above. Mammary tissue was incubated with a cocktail of antibodies including
 607 CD11b-eFluor450 (1:200 eBiosciences #48-0112-82 clone M1/70), CD45-BV510 (1:200
 608 Biolegend #103137 clone 30-F11), Ly6C FITC (1:200 BD Biosciences #553104 clone
 609 AL-21), Ly6G-PE (1:200 Biolegend #127607 clone 1A8), CD11c APC-eFluor780 (1:200

eBiosciences #47-0114-82 clone N418), F4/80 PECy7 (1:200 Biolegend #123113 clone BM8) and Her2 (abcam #ab2428 1:200) antibody. Secondary anti-Rabbit AlexaFluor647 (#A-21245, Invitrogen) was used as secondary for Her2+ cells detection at a 1:200 concentration. Cells were incubated for 30 minutes at 4C in dark, subsequently washed and incubated for 30 more minutes with secondary antibody. PI (V35117, Life Technologies) was used as viability dye in 1:200 concentration. Monocytes were defined as CD45+CD11b+Ly6C+, neutrophils as CD45+CD11b+Ly6G+ and macrophages as CD45+CD11c-F480+CD11b+. CD45- fraction was subsequently analyzed for Her2 expression. CD45-Her2+ cells were sorted. Sorted cells were directly lysed in Trizol LS (Ambion, 10296028) for RNA extraction.

CyTOF analysis

All mass cytometry reagents were purchased from Fluidigm Inc. (former DVS) unless otherwise noted. Mice were injected i.p. with 1mg IdU per mouse 16h prior to the experiment. Lymph nodes were removed and mammary glands were digested using the Miltenyi fatty tissue digestion kit. Cells were then washed with PBS containing 1% BSA and blocked with Fc-blocking reagent (eBioscience) to minimize non-specific antibody binding. Cells were stained with a panel of metal-labeled antibodies against 20 cell surface markers (Fig.5 supplement 1A) for 30 minutes on ice, and then washed. All antibodies were either purchased pre-conjugated to metal tags, or conjugated in-house using MaxPar X8 conjugation kits according to the manufacturer's instructions. After antibody staining, cells were incubated with cisplatin for 5 minutes at RT as a viability dye for dead cell exclusion. Cells were then fixed and permeabilized with a commercial

fix/perm buffer (BD Biosciences) and stored in PBS containing 1.6% formaldehyde and a 1:4000 dilution of Ir nucleic acid intercalator to label all nucleated cells. Immediately prior to acquisition, cells were washed in PBS, and diH₂O and resuspended in diH₂O containing a 1/10 dilution of EQ 4 Element Calibration beads. After routine instrument tuning and optimization, the samples were acquired on a CyTOF2 Mass Cytometer in sequential 10min acquisitions at an acquisition rate of <500 events/s. The resulting FCS files were concatenated and normalized using a bead-based normalization algorithm in the CyTOF acquisition software and analyzed with Cytobank. FCS files were manually pre-gated on Ir193 DNA⁺ CD45⁺ events, excluding dead cells, doublets and DNA-negative debris. Myeloid derived cells were manually gated based on CD11c and CD11b expression and the gated myeloid populations were then analyzed using viSNE³⁷ based on all myeloid phenotypic markers. Putative cell populations on the resulting viSNE map were manually gated based on the expression of canonical markers, while allowing for visualization of additional heterogeneity within and outside of the labeled population bubbles.

Mammospheres and 3D mammary primary epithelial cell cultures

Acini cultures were performed as described^{54,55}. 5×10^4 eCCs were seeded in 400ul Assay Medium in 8-well chamber slides coated with 40ul of Matrigel (Corning). Acini formed at an efficiency of around 30 acini/ 1×10^4 MECs plated. For macrophage co-cultures, primary tissue macrophages were added at a ratio of 500 per 1×10^4 eCCs seeded to 5d old acini cultures. For inhibitory treatments, 5d old acini cultures were treated for 24h with 1 μ M Lapatinib (LC Laboratories), 2uM IKK Inhibitor³⁹ (generous gift

from Dr. Albert Baldwin), 1 μ M CCR2 inhibitor RS504393 (Tocris Bioscience) or DMSO as vehicle control. Cultures were then fixed for immunofluorescence (IF) with 4% PFA. Mammosphere cultures were prepared as described⁵⁵. To prepare conditioned medium, 5d old mammosphere culture were plated in serum free DMEM medium and conditioned medium was harvested after 24h. Elisa detection for CCL2 was done following protocol from manufacturer (catalog number: MJE00, R&D systems).

Immunoblotting and quantitative PCR

Immunoblotting was performed as described previously^{56,57}. Antibodies used were P-NF-kappa-B p65 (Cell Signaling polyclonal) and beta-tubulin (abcam, polyclonal). For expression analysis of FVB wild type mammary epithelial cells or MMTV-Her2 eCCs, epithelial cells were isolated and grown as mammospheres for 5d as described⁵⁵. For expression analysis of Raw264.7 macrophages, cells were grown as monolayers in 6-well culture dishes and treated with conditioned medium for 24h. RNA isolation was performed using Trizol (Life Technologies) or the RNeasy Kit (Qiagen) for MTMs. RT- and qPCR were performed as described⁵⁰. All samples were normalized to GAPDH expression and $2^{-\Delta\Delta C_t}$ values were calculated as described⁵⁸. Primers were purchased from IDT. Primer sequences were: Mouse- GAPDH forward primer 5'-AACTTTGGCATTGTGGAAGGGCTC-3'; GAPDH reverse primer 5'-TGGAAGAGTGGGAGTTGCTGTTGA-3. E-Cadherin forward primer 5'-CAAGGACAGCCTTCTTTTCG-3'; E-Cadherin reverse primer 5'-TGGACTTCAGCGTCACTTTG-3'. Wnt-1 forward primer 5'-CAGTGGAAGGTGCAGTTGCAG-3'; Wnt-1 reverse primer 5'-

679	CAGTGGAAGGTGCAGTTGCAGC-3'.	CSF1	forward	primer	5'-
680	CAACAGCTTTGCTAAGTGCTCTA-3';	CSF1	reverse	primer	5'-
681	CACTGCTAGGGGTGGCTTTA-3'.	CCL2	forward	primer	5'-
682	GGCTGGAGAGCTACAAGAGG-3';	CCL2	reverse	primer	5'-
683	GGTCAGCACAGACCTCTCTC-3'.				

684 **Patient samples.** Paraffin embedded sections from patients with DCIS were obtained
685 from the Cancer Biorepository at Icahn School of Medicine at Mount Sinai, New York,
686 NY. Samples were fully de-identified and obtained with informed consent with
687 Institutional Review Board approval, which indicated that this work does not meet the
688 definition of human subject research according to the 45 CFR 46 and the Office of
689 Human Subject Research.

690 **Circulating Cancer Cell (CCC) and Disseminated Cancer Cell (DCC) detection**

691 For CCC analysis, blood was drawn by cardiac puncture following IACUC protocols.
692 CCCs were purified using negative lineage cell-depletion kit (Miltenyi), fixed with 3%
693 PFA for 20 min on ice and cytopsin preparations were carried out by centrifugation of
694 blood cells at 500 rpm for 3 min using poly-L-lysine-coated slides (Sigma). CCCs were
695 stained by immunofluorescence against cytokeratin 8/18 and HER2. Cytokeratin 8/18
696 and HER2 positive cells were counted and plotted per ml of blood. For DCC analysis in
697 lungs, lung sections were stained against HER2. Complete lung sections were screened
698 for HER2 positive DCCs at 1000x fold magnification. From each animal, the average
699 number of DCC per section from 3 individual lung sections was quantified.

700

Statistical Analysis

Unless noted otherwise, all of the experiments presented in the manuscript were repeated at least 3 times with replicates of at least 3. Statistical Analysis was done using Graph Pad Prism Software. For all cell culture experiments (experiments with technical replicates), one-tailed *student t-tests* were performed. For mouse experiments, *Mann-Whitney* tests were used. Differences were considered significant if *P* values were ≤ 0.05 .

Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request.

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Author Contributions.

N.L. M.S.S and M.C.A designed and optimized experimental approach, performed *in vitro* and *in vivo* experiments, analyzed data and co-wrote the manuscript, A.M. and A.R. and the Human Immune Monitoring Core performed CyTOF experiments, J.J. and J.C. did TMEM histopathology and scoring, E.F., E.T., and K.H. performed experiments, M.M. provided general guidance and oversight and co-wrote the manuscript, J.A.A.-G. designed and optimized experimental approach, analyzed raw data and provided general guidance and oversight, analyzed data and co-wrote the manuscript.

905

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921 **Author information.**

922 The authors declare no competing financial interests.

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FIGURES

Figure Legends.

Figure 1: Macrophages enter the ductal epithelial layer in early breast cancer lesions. H&E staining of mammary gland sections show progression from healthy mammary ducts in FVB wild type glands (age 20wks; **a**) to early lesions classified as hyperplasia and mammary intra-epithelial neoplasia (age 22wks; **b**) to invasive tumors (age 26-30wks; **c**) in the MMTV-HER2 mouse model. Bars: 100µm Mammary glands from FVB wild type (20 weeks) (**d**) of pre-malignant MMTV-HER2 mice at age 14 (**e**) and 22 (**f**) weeks were stained against F4/80 (macrophages) and CK8/18 (epithelial cells) and against F4/80 and SMA (**g-i**). Bars: 10µm. The mean plus SEM of the percentage of ducts containing IEM is shown; FVB: N=4 mice, 14wks N=3 mice, 22wks N=5 mice (**j**). P values were calculated with 95% confidence by Mann-Whitney test.

Figure 2: Intra-epithelial macrophages induce an EMT-like response in early cancer cells. 20wk old MMTV-HER2 mouse mammary glands were stained against E-Cadherin and F4/80. E-Cadherin localization was analyzed dependent on whether macrophages did not make direct contact to the duct (no M. or distant M.; **a**) or whether ducts contain intra-epithelial macrophages (IEM; **b**). The percentage of individual epithelial cells with disrupted E-Cadherin was quantified in 4 mice and is shown as mean plus SEM (**c**). Statistical test: Mann-Whitney. E-Cadherin mRNA expression in whole mammary glands of FVB wild type (WT, 20wk, N=4) or 20wk old MMTV-HER2

(N=7) mice was determined by qPCR and is shown as mean plus SEM, Mann-Whitney test (d). 24wk old MMTV-HER2 mammary glands (N=3 per group) were stained against β -Catenin and Iba1, a macrophage marker (e,f). β -Catenin+ early cancer cells (blue) were more frequent in ducts containing intra-epithelial macrophages IEM (g). Plots show mean plus SEM, Mann-Whitney test. The mammary epithelial cell line Comma-1D was grown as a monolayer before addition of mCherry expressing Raw264.7 macrophages E-Cadherin (h) or β -Catenin (i) was stained and the intra-nuclear signal intensity of β -Catenin was quantified (j). Plots show nuclear β -catenin signal intensity in individual cells; independent experiments N=3, student's t-test. (k,l) Conditioned medium was harvested from primary mammospheres of 20-22wk old wild type (WT) or pre-malignant MMTV-HER2 mice and added to Raw264.7 macrophages or mammary tissue macrophages (MTMs) isolated from pre-malignant MMTV-HER2 mammary glands. Wnt-1 expression is depicted as mean plus SEM of three technical replicates. Statistical significance was determined by Student's t-test with 95% confidence; individual experiments N=3 for Raw264.7 and N=2 for MTMs. Comma-1D cells were grown as monolayers (m) and Raw-264.7-mCherry macrophages were added (n) and additionally treated with DKK1 (o). E-Cadherin signal intensity in whole section was quantified and is shown as mean plus SEM where each dot represents one microscopic field; independent experiments N=2, Student's t-test. The pixel intensity of the cells in n) is background pixel signal for the green channel, ~1000 in pixel intensity.

Figure 3: Macrophage depletion during pre-malignant stages prevents early cancer cell dissemination. 20wk old pre-malignant MMTV-HER2 mice were treated

with the anti CSF1R ASF98 antibody and animals were harvested after two weeks with no signs of invasive carcinoma (**a**). Analysis of HE staining of mammary gland sections confirmed the absence of invasive lesions (**b,c**; bars 100 μ m). E-Cadherin expression in whole mammary glands was determined (**d**; mean plus SEM; control 7, anti-CSF1R 6 animals; Mann-Whitney test) and by immunofluorescent staining against E-Cadherin in mammary gland sections (**e,f**; bars 10 μ m). E-Cadherin signal intensity was measured in individual regions of cell junctions in 3 animals per group (**g**). p-values were calculated with 95% confidence by Mann-Whitney test. Early circulating cancer cells (eCCCs) were quantified in mice per group as the amount of HER2 and CK8/18 positive eCCs per mL peripheral blood. Values were normalized to the mean of controls and are shown as mean plus SEM (**h**) depicts normalized means plus SEM; 7 animals per group; Mann-Whitney test. Disseminated HER2+ eCCs were quantified in lung sections (**i,j**; bars 25 μ m) and were quantified as the average of HER2+ cells per 100 randomly chosen microscopic fields (**k**). Means + SEM are shown, Mann-Whitney test was used for statistical analysis.

Figure 4: Early disseminated cancer cells contribute to metastasis formation.

Macrophages were depleted from pre-malignant MMTV-HER2 mice by ASF98 treatment starting at week 18. Treatment was stopped when mice developed palpable tumors (1-3mm average) (**a**). Mice were left until tumors reached 1cm in diameter and then sacrificed. **b**) Time from beginning of treatment at age wk18 until development of palpable tumors as mean plus SEM (9 mice each, 23-38 wk old). **c**) Time from formation of palpable tumors until tumors were overt (26-43 wks old) as mean plus SEM

(Control N=9, anti-CSF1R N=6). Macrophages in sections of overt tumors (at least 3 animals per group) were identified by staining against F4/80 at the end of the experiment (**d,e**; bar 100 μ m) and quantified as numbers of macrophages relative to tumor area (**f**; statistical analysis Mann-Whitney). Vascularization of overt tumors was analyzed by staining against endomucin, an endothelial cell marker (**g,h**; bar 100 μ m) and quantified as endomucin+ area / tumor in at least 3 animals combined (**i**; mean plus SEM; Mann-Whitney). Solitary DCCs in lung sections and metastases defined as cell clusters bigger than 3 cells were quantified in lung sections stained against HER2 (**j-l**, bar 25 μ m) For solitary cell analysis, the average of DCCs or metastases per 100 fields was counted; each dot represents one lung section. For metastasis analysis, the total number of metastases per lung sections was quantified and plotted (**j**). Number of mice N=6 (control) and N=4 (α CSF1R) animals combined; statistical tests: Mann-Whitney.

Figure 5: Phenotypic profiling of immune cells in early mammary cancer lesions.

a) Whole mammary glands from FVB wild type mice or 14 and 22 week old pre-malignant MMTV-HER2 mice were analyzed by mass cytometry. viSNE plots were generated from myelo-monocytic cells (gating strategy see **Supplementary Fig.4a-b**). Results from one representative animal is shown; number of animals per group N=5; individual experiments N=2. The three subpopulations were identified as Ly6C+ monocytes and CD206-hi and CD206-lo macrophages based on their expression levels of Ly6C (**b**) and CD206 (**c**). These three populations were then analyzed for their frequency amongst all myelomonocytic cells (**d-f**) Dot plots show mean plus SEM of 5 animals per group. Heat plots for 3 individual animals per group with expression levels

of selected markers Ly6C (**b**), CD206 (**c**), Tie2 (**i**) and IdU incorporation as a proliferation marker (**j**) were generated for CD206-lo and -hi macrophages as identified in the viSNE plots. **g, h**: viSNE plot and quantification of myelomonocytic population in overt MMTV-HER2 tumors (5 mice per group). Mammary glands from FVB wild type mice (**k**, 22wks), MMTV-HER2 mice at 24wks (**l**) and overt MMTV-HER2 tumors (**m**, 26-30wks) were stained against CD206 and F4/80 and CD206 signal intensity in F4/80+ macrophages was quantified. Plot (**n**) depicts mean plus SEM, each dot represents one macrophage; 3 animals combined. All bars: 10 μ m. All statistical testing was done with Mann-Whitney with 95% confidence.

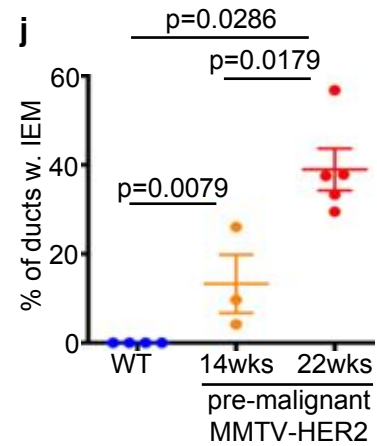
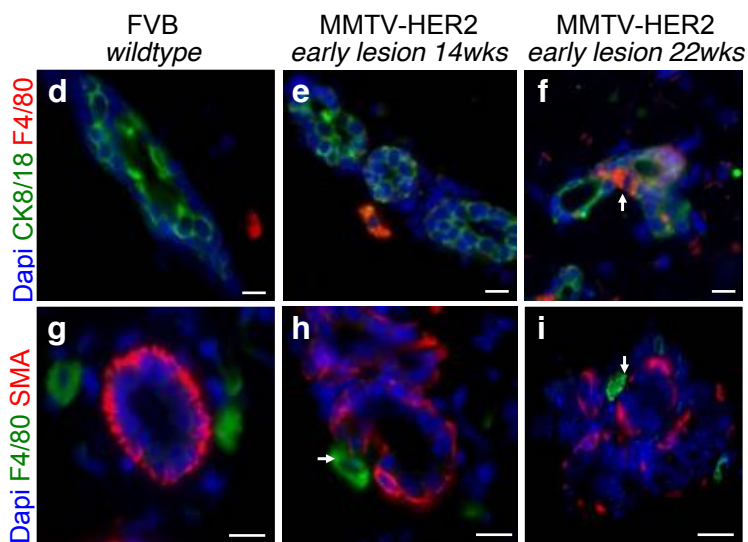
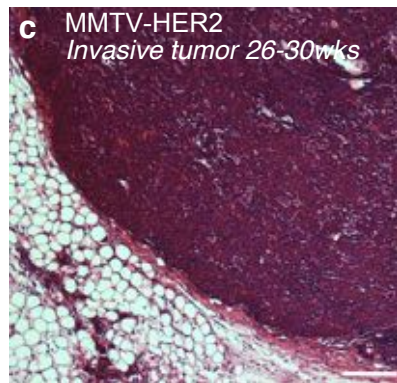
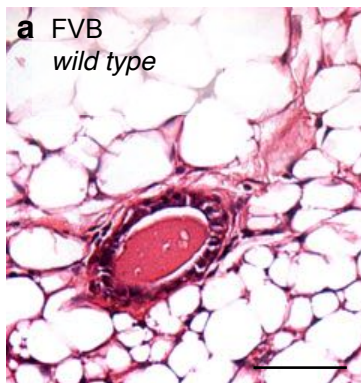
Figure 6: HER2 activates NFkB and upregulates CCL2. **a)** Western blot for NFkB subunit phospho-p65 in mammosphere (MS) lysates from 20wk old MMTV-HER2 mice. One representative blot of three independent experiments is shown. **b)** CCL2 expression in MS from FVB wild type and pre-malignant MMTV-HER2 mammary glands MG. Technical replicates, 3 experiments (3 mice/group), statistics: Mann-Whitney, 95% confidence. **c)** MG from FVB wild type and 22wk old MMTV-HER2 mice stained against CCL2, HER2, and CCR2; Bars 25 μ m. Insert, CCR2 signal; full image in Supplementary Fig.5i. CCL2 intensity was quantified using ROI tool in Metamorph (**Supplementary Fig5h**). **d)** ELISA of CCL2 in 24 hrs conditioned media of wt or HER2+ MS isolated from 2 animals. p value Mann-Whitney test, 95% confidence – SEM shown. **e-g)** CCL2 staining in acini cultures from MMTV-HER2 MG (20-24wks) grown for 5d and then DMSO-treated (vehicle control; **e**), 1 μ M Lapatinib (**f**) or 1 μ M IKKi compound A (**g**) for 24h. Bar 25 μ m. **h-j)** MG acini treated with DMSO (vehicle), Lapatinib (1 μ M), IKKi

compound A (1 μ M), or CCR2 inhibitor RS504393 (1 μ M). Primary MG macrophages were then added and percentage of acini associated with or without macrophages (**h**) was quantified (**i**; bar 25 μ m); (**j**): means plus SEM; each dot one technical replicate; 2 independent experiments; statistics: Mann-Whitney - SEM shown). **k-m**) 20wk old MMTV-HER2 mice were treated with CCR2 inhibitor RS504393 (2mg/kg i.p. daily) for 2 weeks and stained against E-Cadherin and F4/80 (**k,l** bars 25 μ m). **m**) Intra-epithelial macrophage (IEM) containing ducts were quantified (Mann-Whitney, 95% confidence; each dot: mean plus SEM of one animal). **n**) Quantification of circulating cancer cells (CCC) in the mice in the experiment in k-m (statistics Mann-Whitney - SEM shown). CCC quantification was performed as in Fig3h. **o**) 22wk old MMTV-HER2 mice were treated locally with 2mg/kg CCR2 inhibitor injected into the MG fat pad every day for 5 days and vehicle control into the contra-lateral gland. Glands were stained against F4/80 and intra-epithelial macrophage (IEM) containing ducts were quantified (**o**: 5 mice per group, statistical test Mann-Whitney - SEM shown). Values in o) normalized to the IEM content of each contra-lateral control treated gland.

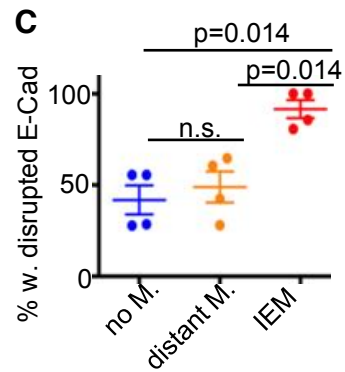
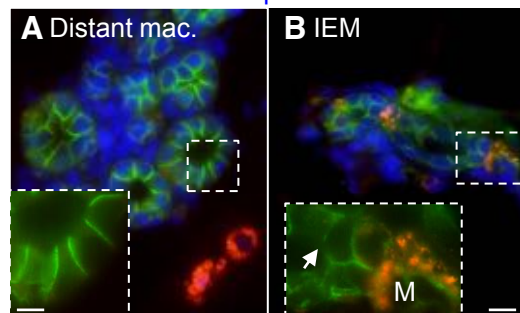
Figure 7: Intra-epithelial macrophage numbers in human DCIS lesions negatively correlate with E-Cadherin levels. Human adjacent healthy (**a**) and DCIS tissue (**b**) was stained against CD68 (macrophages) and smooth muscle actin (SMA). Bar: 75 μ m. **c**) Plot shows mean plus SEM of the percentage of ducts intra-epithelial macrophages (IEM) from 7 healthy and 10 DCIS patients; each dot represents one patient, statistical test: Mann-Whitney. Sections from human DCIS tissue were stained against CD68 (macrophages) and E-Cadherin (**d,e**, bar: 75 μ m). E-Cadherin pixel intensity was

quantified in regions of individual cell junctions and medians for individual patients were quantified. Plot (f) depicts mean E-Cadherin intensity throughout DCIS lesions of individual patients with low or high intra-epithelial macrophage (IEM) numbers (total patient number N=12, statistical test Mann-Whitney - SEM shown).

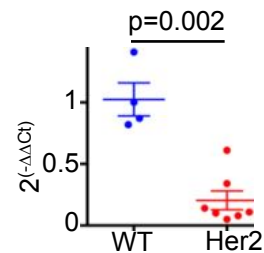
Figure 8: Models summarizing findings and potential scenarios for early DCC role in metastasis development. a) Scheme of macrophage-regulated early dissemination from pre-invasive lesions where CCR2⁺/CD206^{hi}/F4/80^{hi}/Tie2^{hi} macrophages are attracted into early lesions by HER2 and NFkB mediated upregulation of CCL2. Our models supports that intra-epithelial macrophages in turn secrete Wnt-1 in response to CCL2 production by cancer cells but also other immune cells and thereby further cement an EMT-like response that is also stimulated by autocrine/paracrine production of other Wnt ligands^{8,9} to drive early dissemination. b) eDCCs contribute to metastasis formation, either as a slow cycling seeds of metastasis (scenario 1) or by interacting with the microenvironment and/or later arriving DCCs to create a “pre-macro-metastatic niche” that is more permissive for the growth of late or early and late cancer cells (scenario 2).



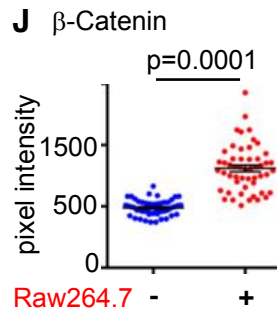
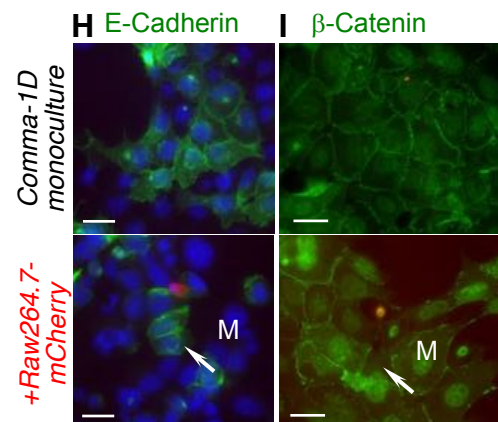
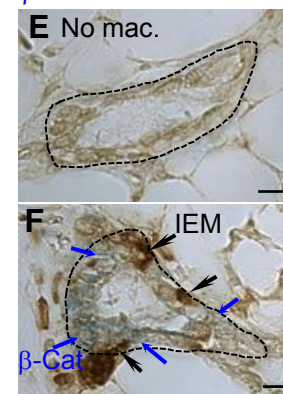
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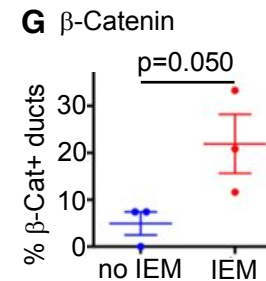
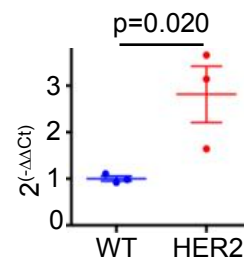
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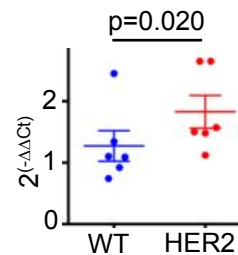
β-Catenin Iba1



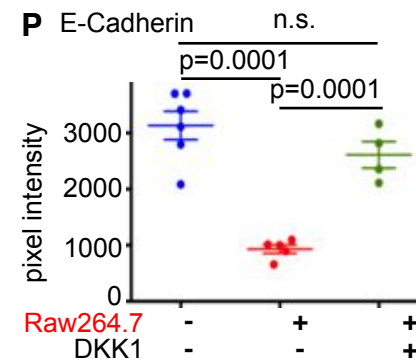
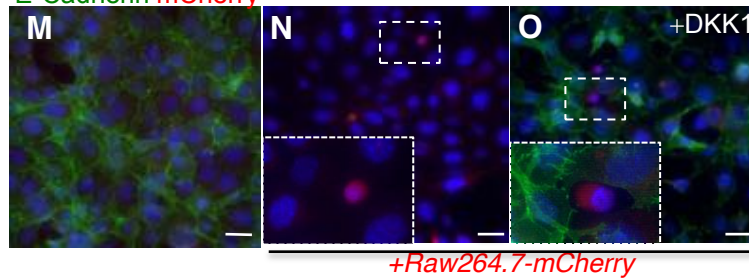
K qPCR Wnt-1 in Raw264.7

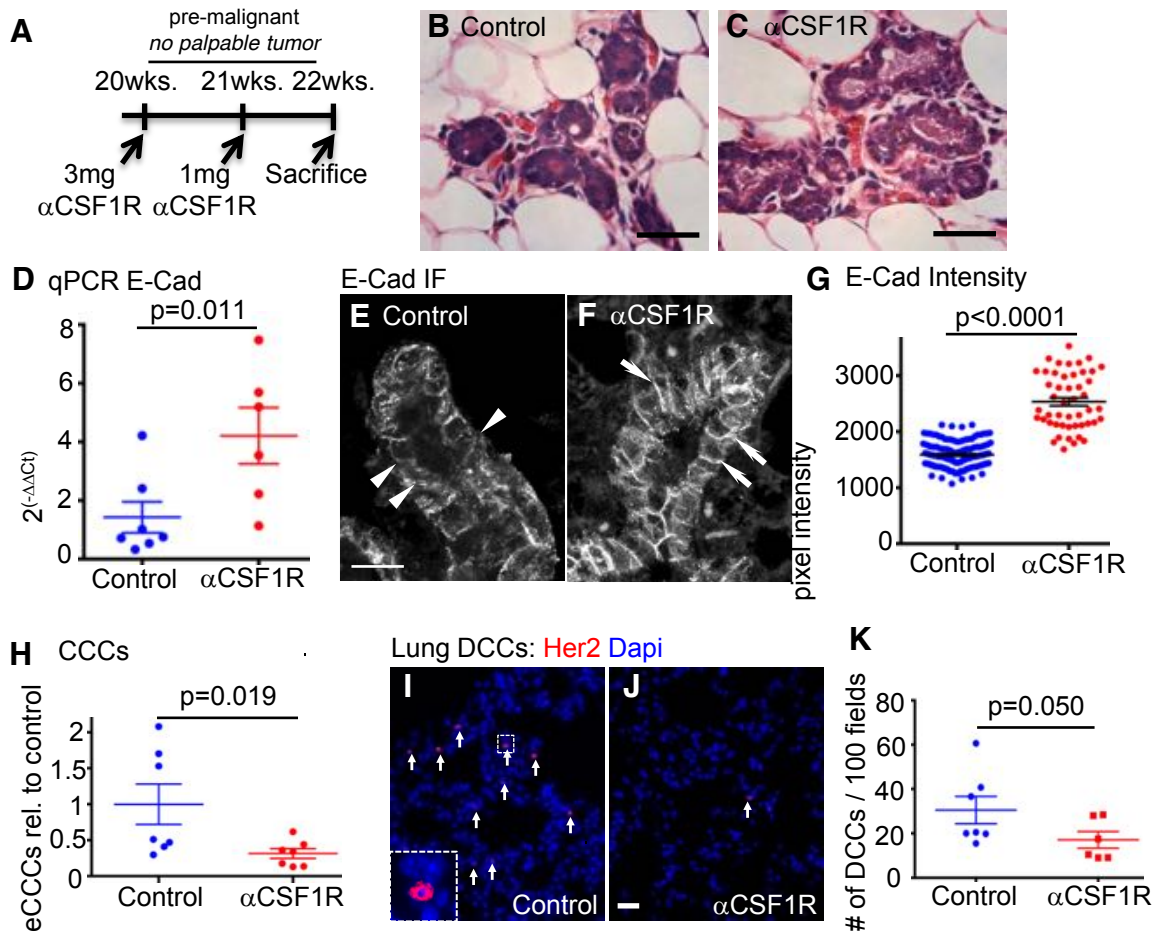


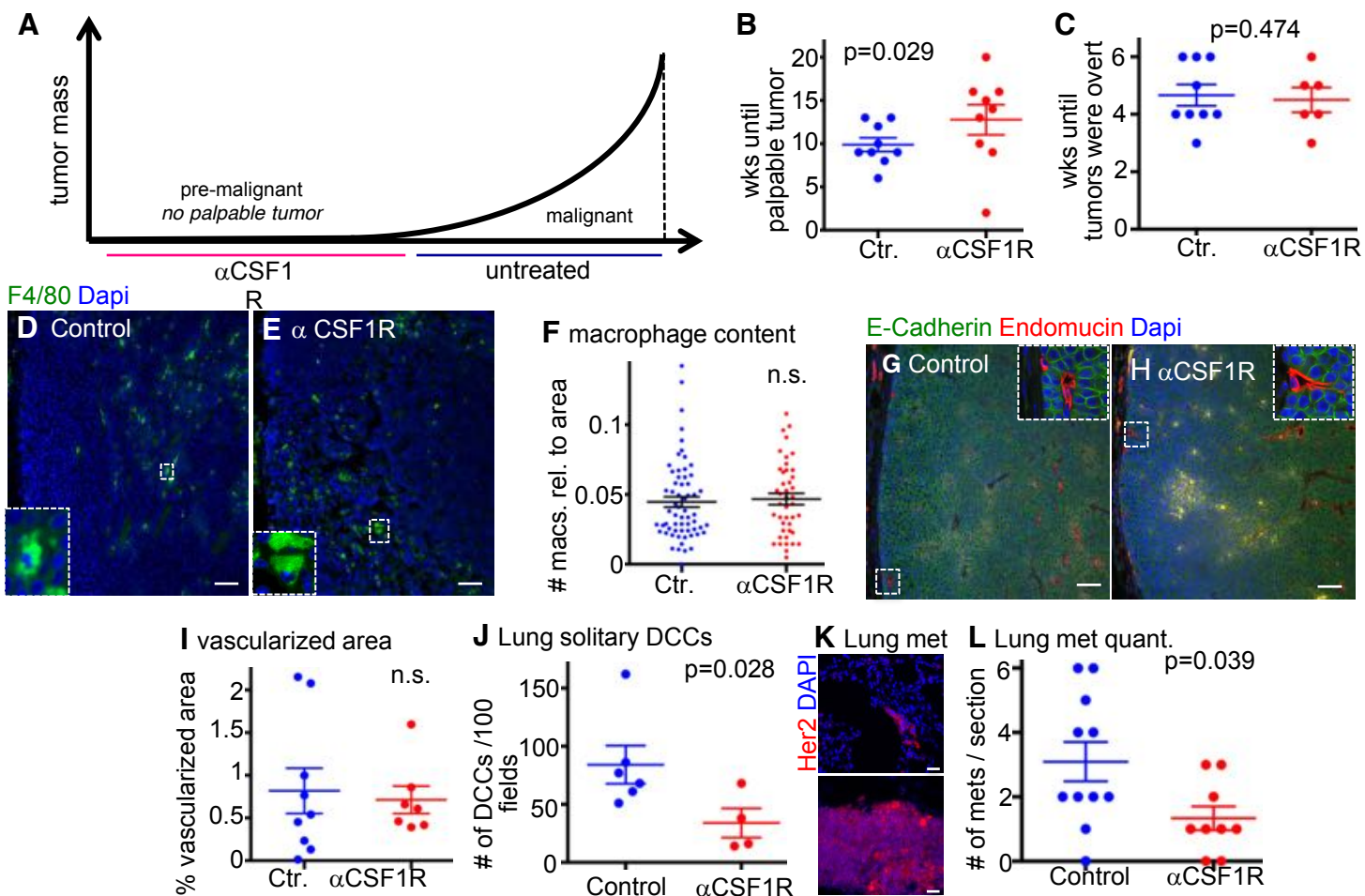
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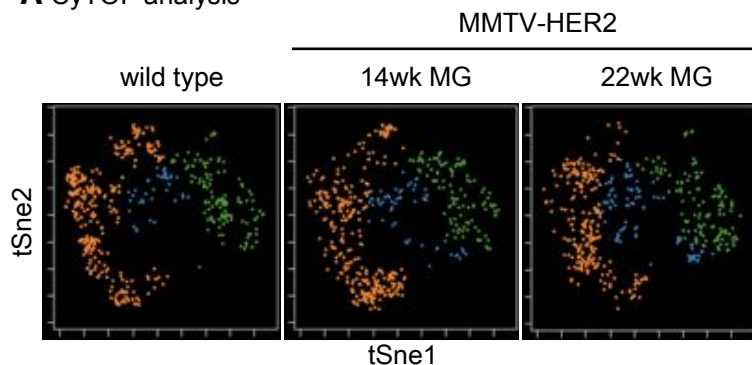
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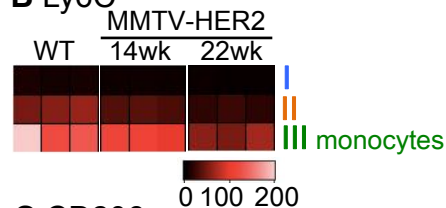




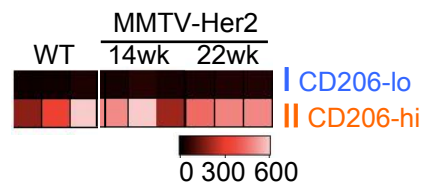
A CyTOF analysis



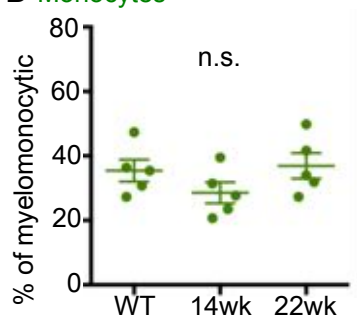
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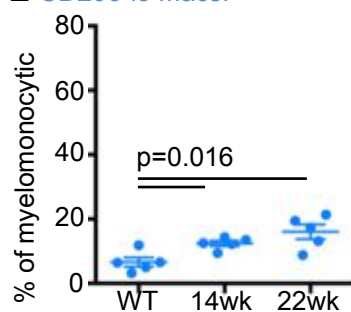
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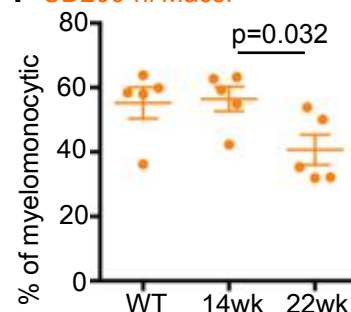
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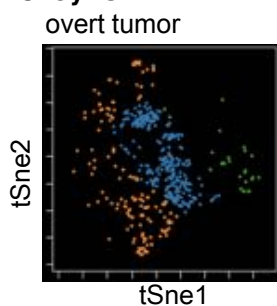
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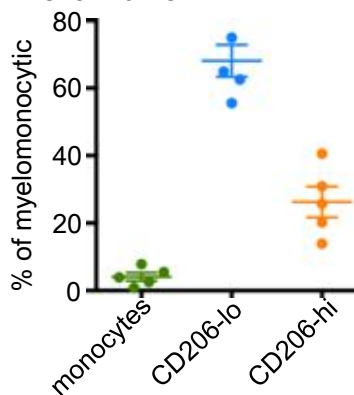
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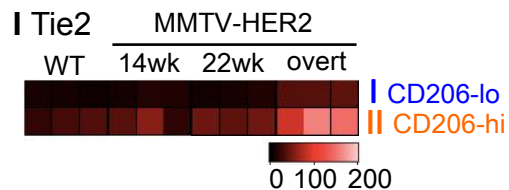
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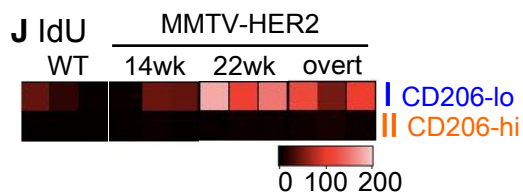
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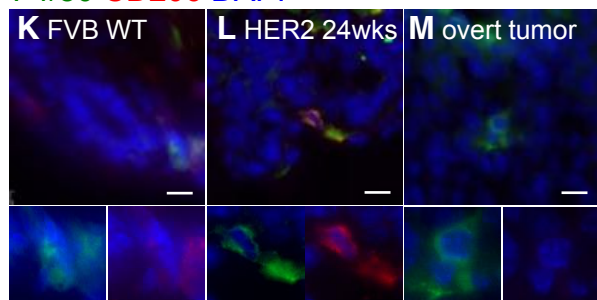
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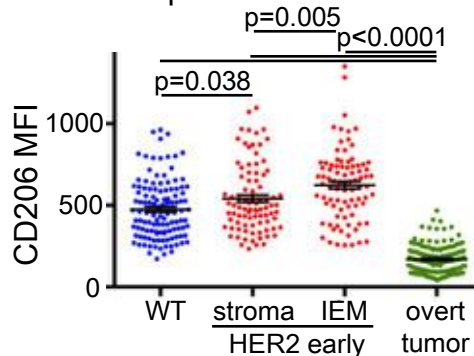
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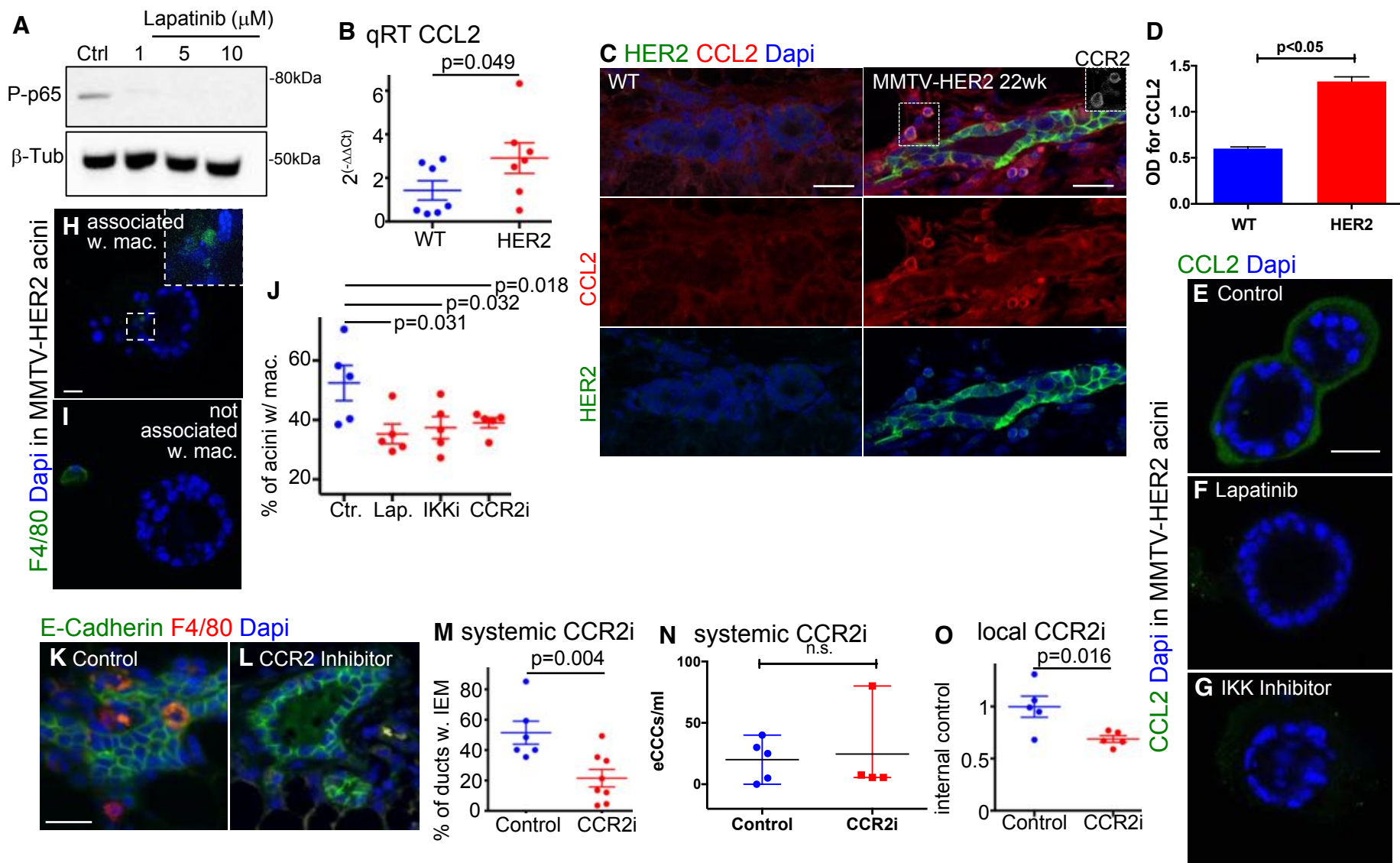


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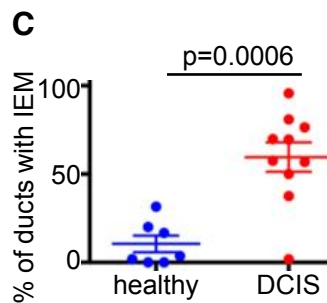
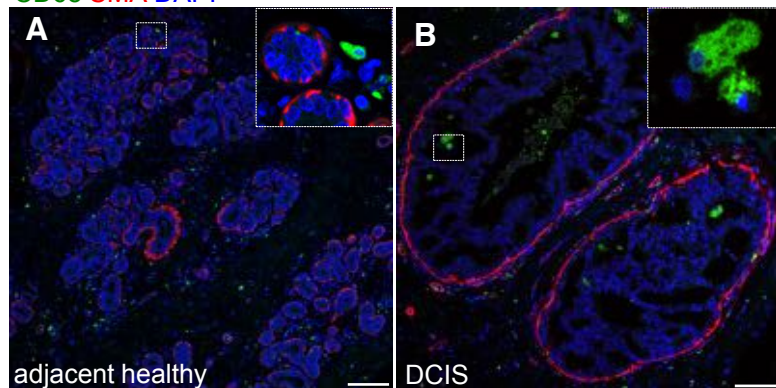


N CD206 quantification

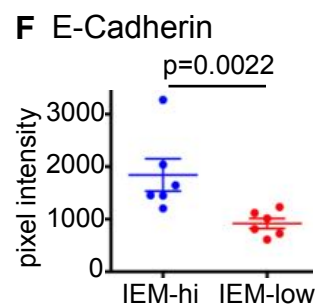
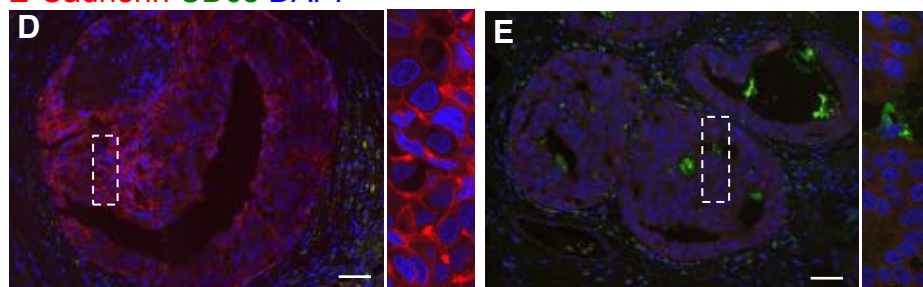




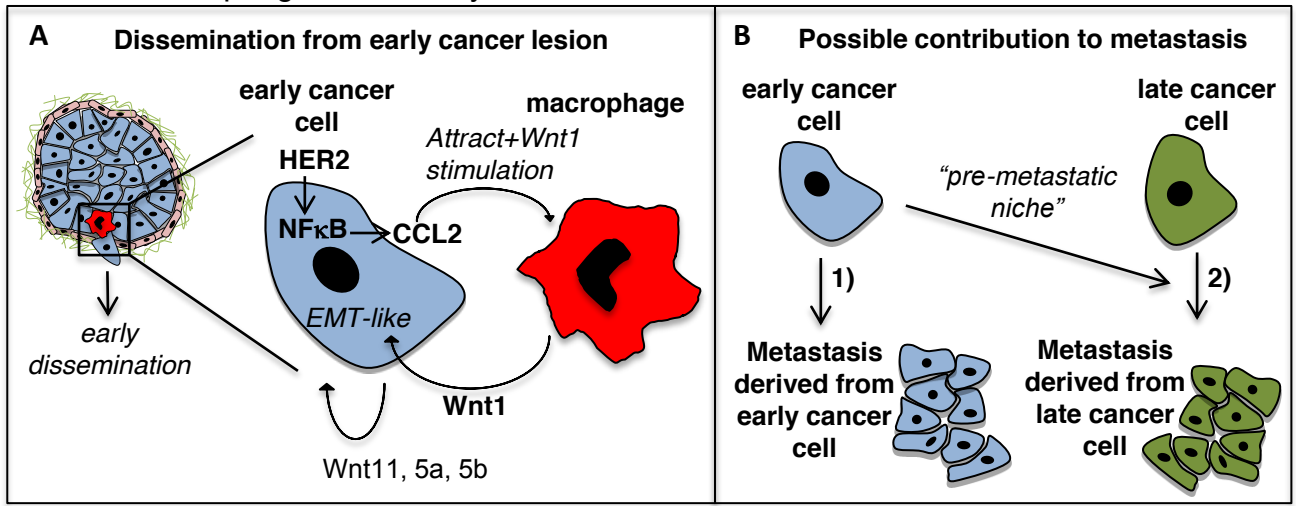
CD68 SMA DAPI

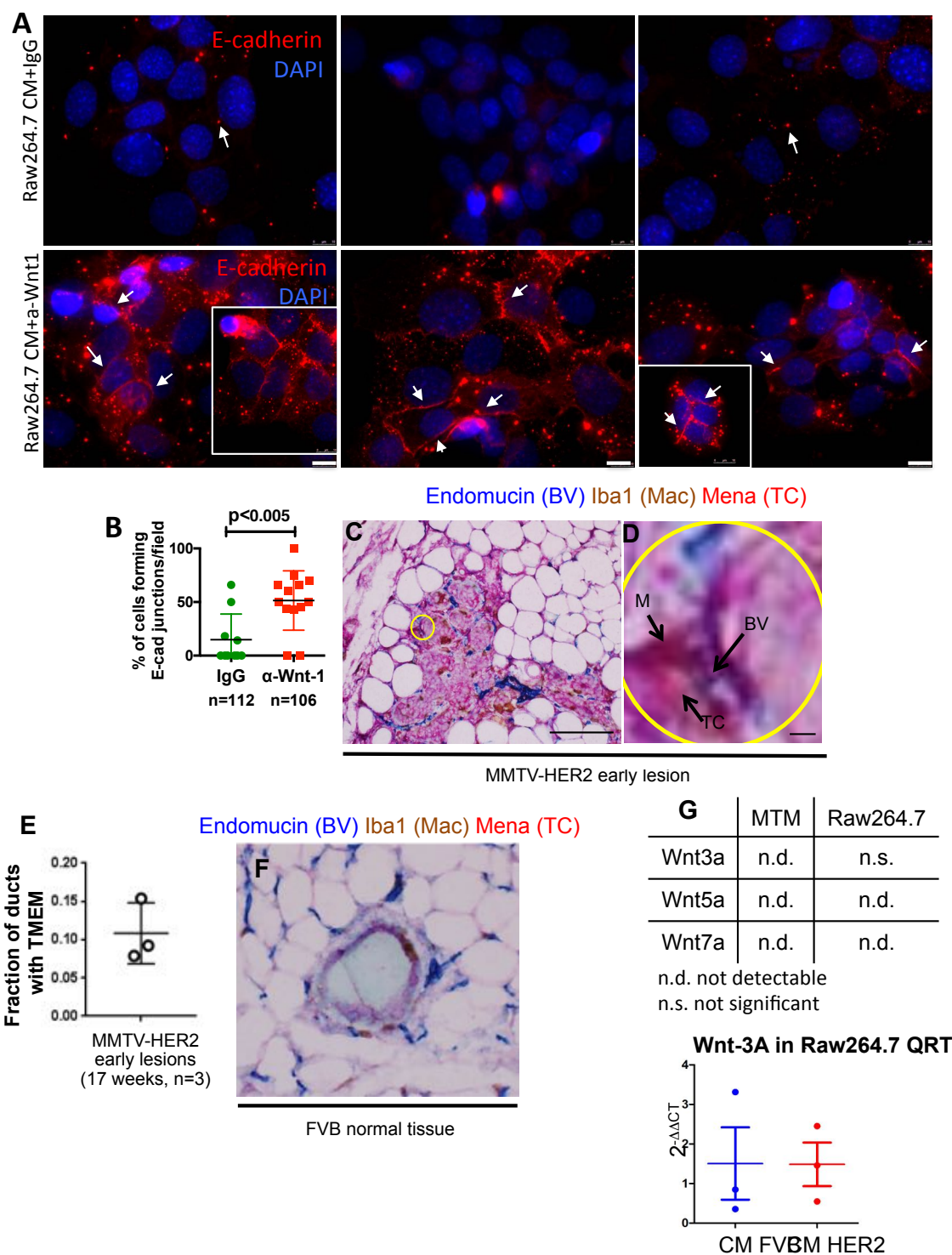


E-Cadherin CD68 DAPI

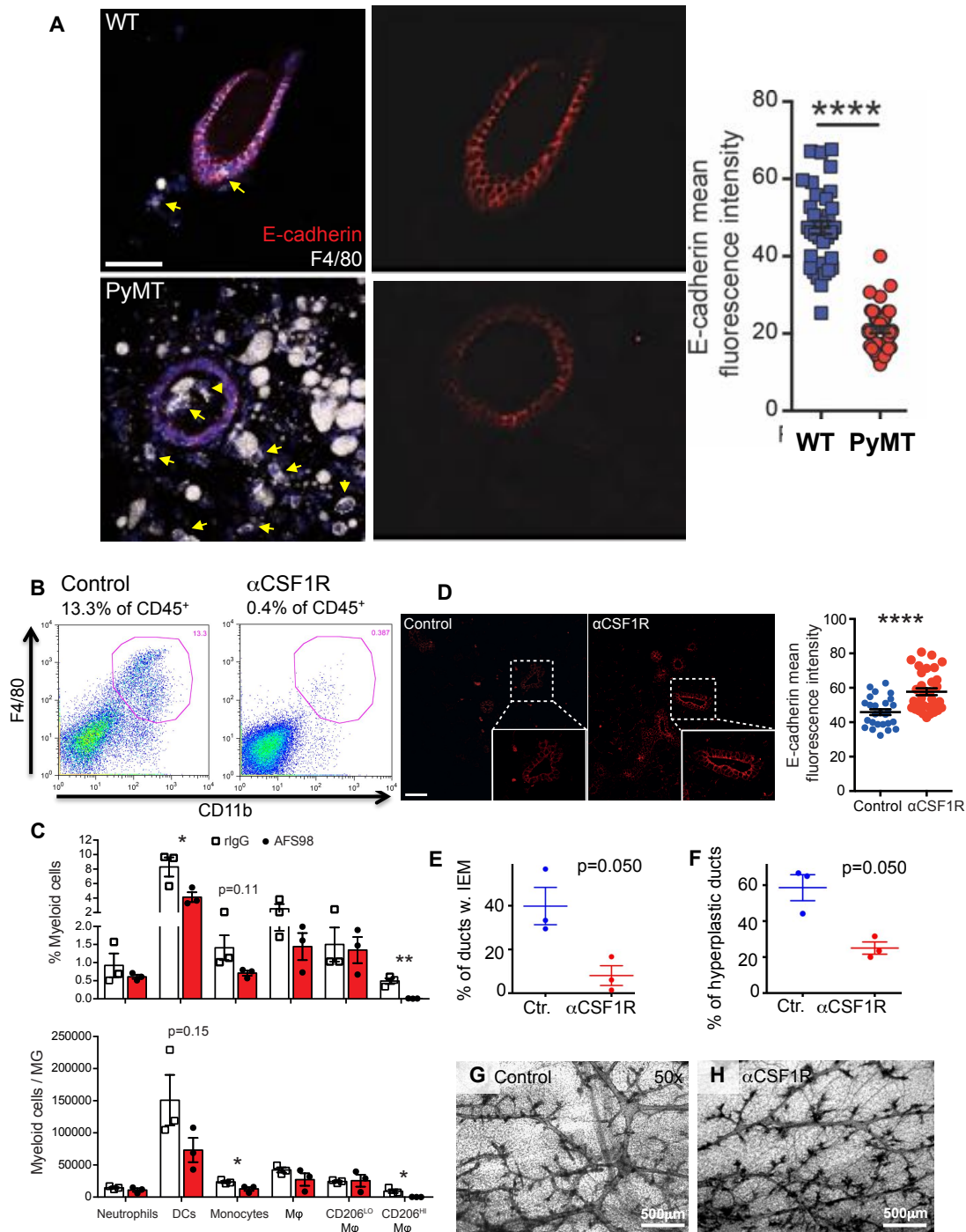


Model of macrophage driven early dissemination

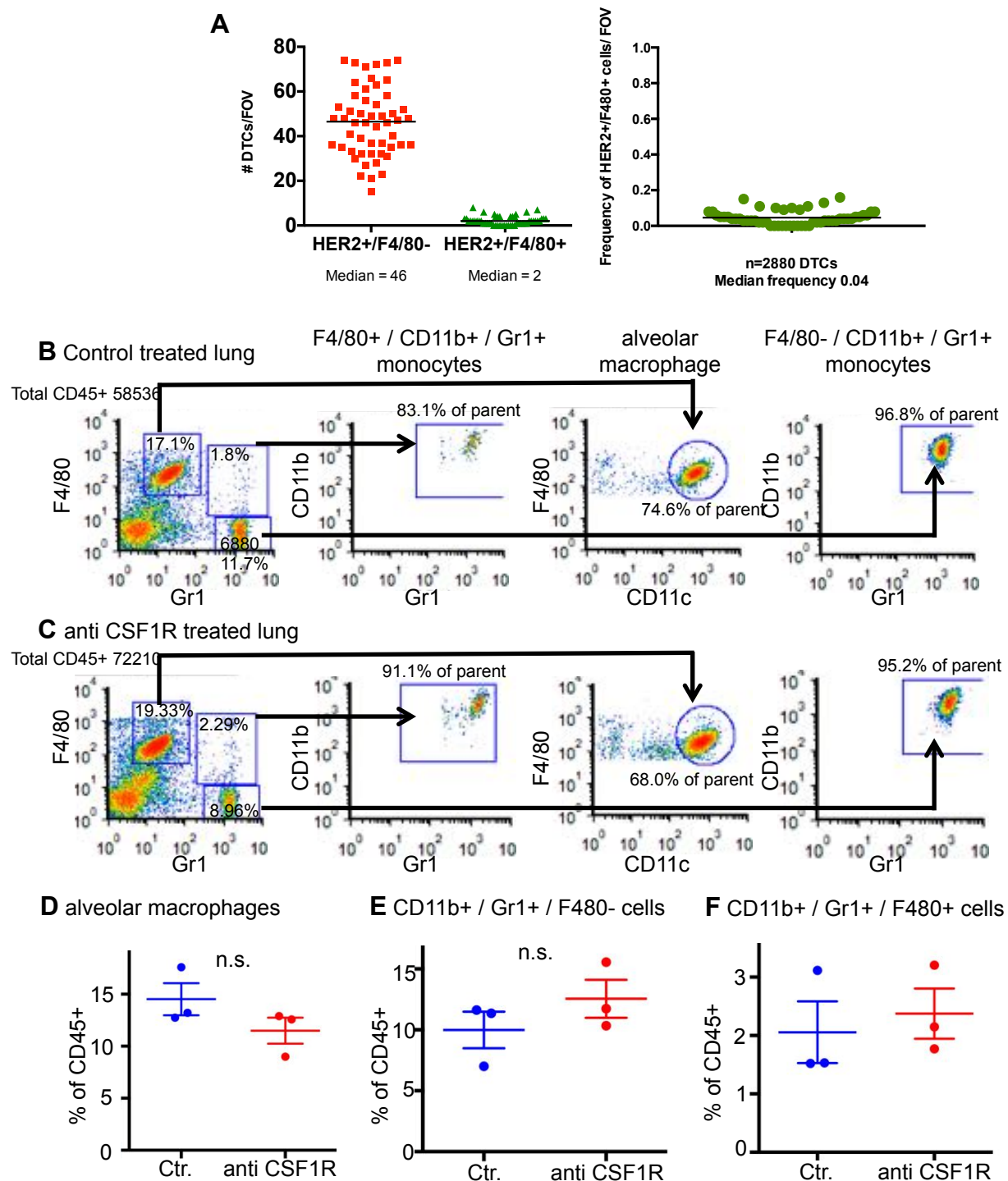




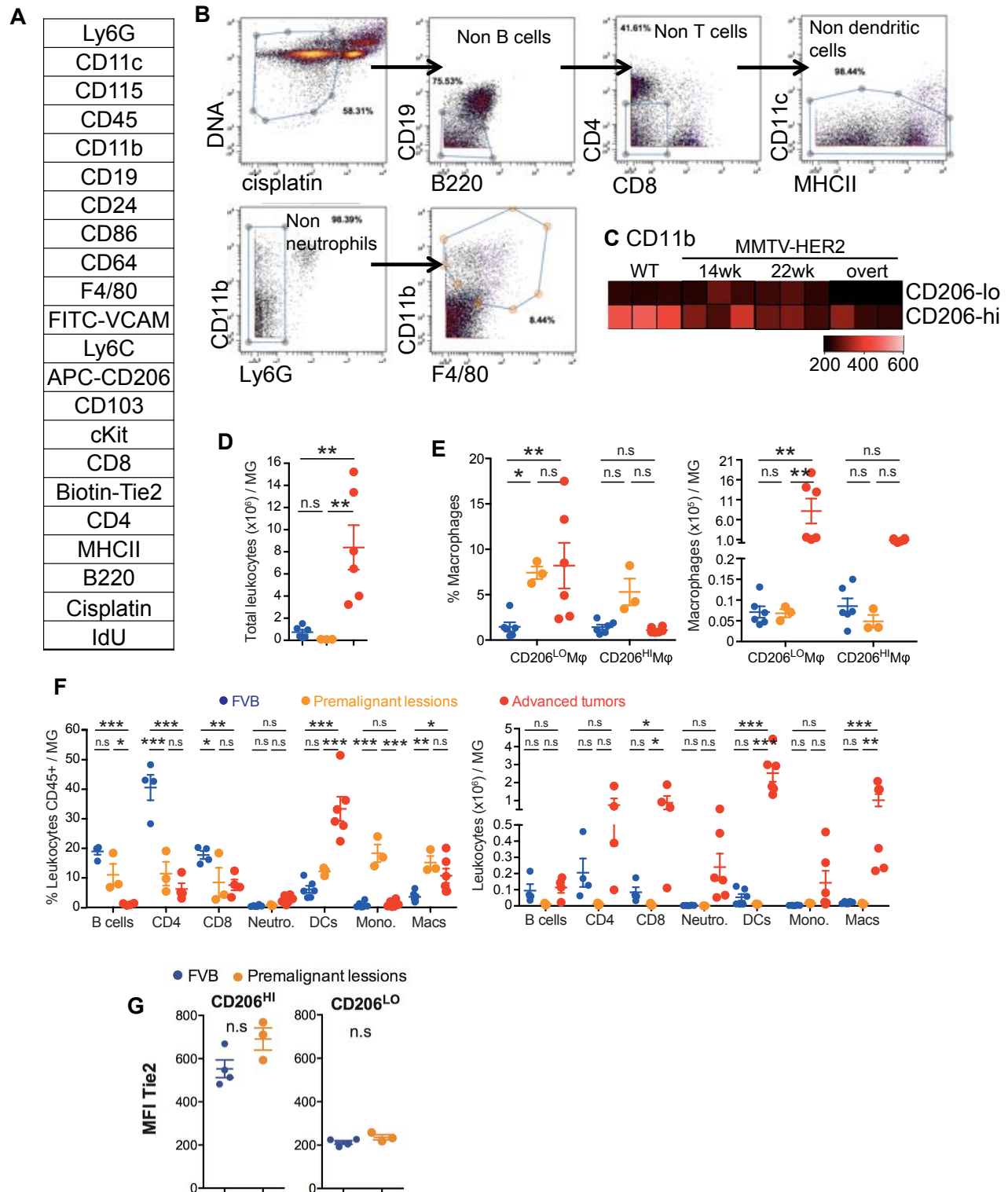
Supplementary Figure 1. Macrophage secreted Wnt1-induced loss of E-cadherin junctions and TMEM formation in early lesions. **a:** IF detection of E-cadherin junctions in Comma-1D cells cultured for 24 hrs in the presence of conditioned media from RAW264.7 monocytic cells treated with an IgG control or with an antibody specific to mouse Wnt1 to deplete non-specific or Wnt1 proteins. Arrow point to globular E-cadherin not engaged in junctions in the top row and junctional E-cadherin in the bottom row. Bars 10 μ m **b:** quantification of the results in A showing the percent of DAPI+ cells forming E-cadherin junctions. n, number of scored cells. P-value calculated with Mann-Whitney test at 95% confidence. Detection of TMEM in MMTV-HER2 early lesions (c) and the yellow circle area showing an example of TMEM is magnified in d. Endothelial cells (BV) are labeled by endomucin blue, early cancer cells by MENA staining (TC) cells in pink/red and macrophages by IBA1 staining in brown (Mac). Arrows in D points to the indicated cell types Mac: M. scale bars: c 100 μ m, d 10 μ m. **e:** quantification of the frequency of TMEM/early lesion ductal structure. Note that the frequency of 10% is in range with the detection of intraepithelial macrophages in Figure 1J in 14 weeks early lesions.



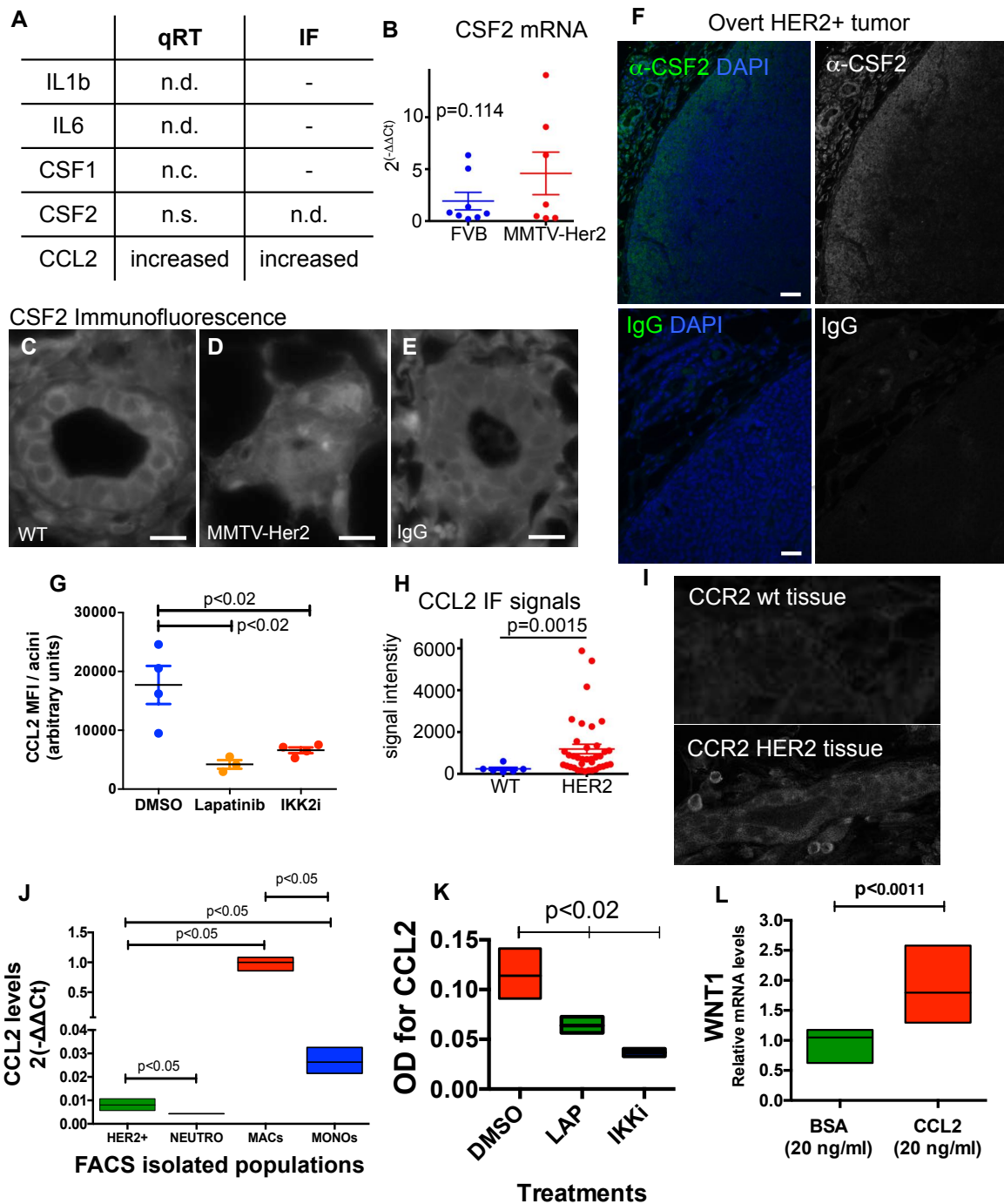
Supplementary Figure 2. E-cadherin junctions in early MMTV-PyMT lesions and effect of macrophage depletion on early lesion immune and epithelial profile. **a:** Immunofluorescence of wt (top) and MMTV-PyMT early lesions (bottom, 11 weeks old) showing macrophages (F4/80, white) and E-cadherin (red). Yellow arrows point to macrophages. Scale bar is 50 μ m. Quantification of E-cadherin mean fluorescence pixel intensity is shown in wt and MMTV-PyMT on the graph on the right. N=26-32 ducts. p value<0.001 calculated with Mann-Whitney test at 95% confidence. **b:** FACS measurement of mammary gland macrophage depletion (F4/80+/CD11b+) after two weeks of treatment with a CSF1R antibody compared to vehicle control (pre-malignant MMTV-HER2 20wks at start of treatment, 3 mice per group). **c:** Flow cytometry quantification of myeloid populations among CD45+ leukocytes in the mammary gland of wt rIgG (white bars) and AFS98-treated (red bars) of MMTV-Her2 females. N=3 females per group; * p $\leq$ 0.05 paired t test. Plots represent mean values $\pm$ SEM. **d:** Representative immunofluorescence images showing E-cadherin signal for depletion experiment shown in C. Scale bar: 50 μ m. Quantification of mean fluorescence intensity per duct is shown on the right graph. ****p $\leq$ 0.0001 calculated by Mann-Whitney. **e:** Percentage of ducts with intra-epithelial macrophages (IEM; **e**) and hyperplastic ducts (**f**) was quantified in mammary gland sections (3 mice per group) after two weeks of CSF1R antibody or vehicle treatment shown in b. **g-h:** Whole mount staining of mammary glands after two weeks of vehicle control (**g**) and CSF1R antibody (**h**) treatment. Scale bars are shown for each panel.



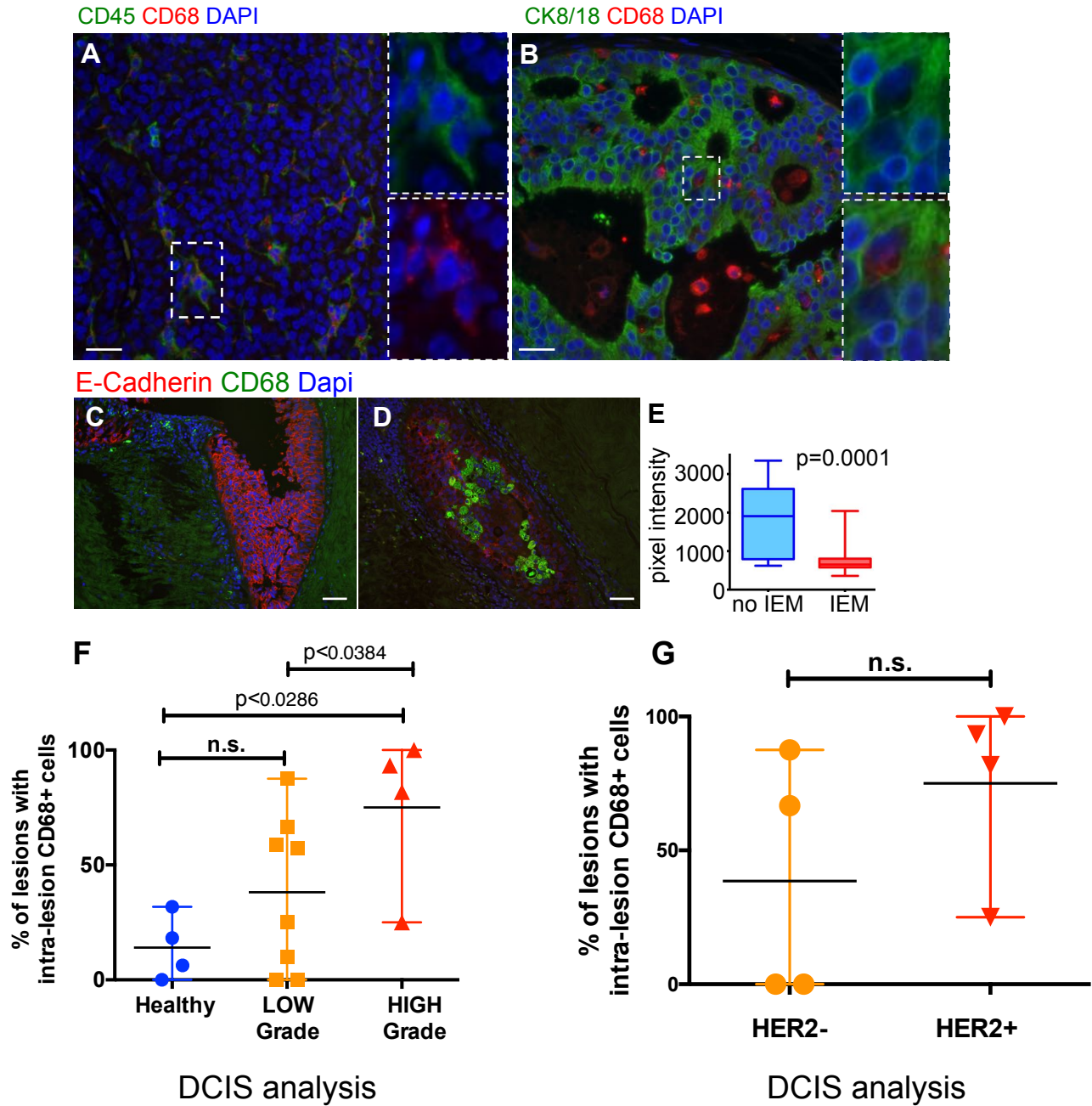
Supplementary Figure 3. Controls for macrophage engulfment of early HER2+ DCCs and gating strategy for macrophage subtype analysis. **a:** Quantification of HER2+/F4/80- and HER2+/F4/80+ cells in lung sections at 40X magnification; 60 fields were scored. Note that majority of cells (96%) when HER2+ are F4/80-. **b, c:** Flow cytometry lung gating strategy were CD45+ viable cells were identified as F4/80+/Gr1+/CD11b+ and F4/80-/Gr1+/CD11b+ monocytes and F4/80+/Gr1-/CD11c^{hi} alveolar macrophages. Quantification of alveolar macrophage (**d**) and monocyte populations (**e,f**) in lungs of MMTV-HER2 mice (3 per group) that had been treated with a CSF1R antibody or vehicle control during pre-malignant stages and were then allowed to progress to over tumor stages (Fig.4). Statistical testing: Mann-Whitney with 95% confidence.



Supplementary Figure 4. Immune profiling of early lesions using CyTOF and multi-color FACS. **a:** List of markers analyzed by mass cytometry (CyTOF). **b:** Gating scheme to create viSNE plots of macrophages and monocytes. DNA staining and Cisplatin was used to identify viable cells. **c:** Heatplot of CD11b in the three myelo-monocytic populations identified by visne plot (Fig.2A). N=3 animals per group; independent experiments: N=2. **d:** CD45⁺ leukocytes in wt (blue), early lesions (yellow) and overt tumors (red) in mammary glands of MMTV-Her2 females. N=3-5 mice per group. n.s., not significant; ** $p \leq 0.01$. Plots represent mean values $\pm$ SEM. **e:** Frequency (left) and absolute number (right) of macrophages subsets CD206^{LO} and CD206^{HI} among CD45⁺ leukocytes. Mφ, macrophage. Plots represent mean values $\pm$ SEM. **f:** Frequency of lymphoid and myeloid cells among CD45⁺ leukocytes (left) and absolute numbers (right) in the mammary glands (MG) shown in E. Neutro, neutrophils; DCs, dendritic cells; mono, monocytes; macs, macrophages. n.s., not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Statistical test: 2 way ANOVA. Plots represent mean values $\pm$ SEM. **g:** Mean fluorescence intensity of Tie2 for CD206^{HI} and CD206^{LO} macrophage subsets in wt and MMTV-Her2 females early lesions (20-24wks, 3-5 mice per group) as measured by multi-color FACS.



Supplementary Figure 5. Analysis of macrophage recruiting cytokines in early lesions. a: Results for qRT-PCR expression and immunofluorescent analysis results of selected cytokines in mammospheres derived from 20wk old MMTV-Her2 mammary glands that were not detectable (n.d.), did not change (n.c.) or did not change significantly (n.s.) or were increased in relation to expression levels in FVB wild type mammary glands. b: qRT-PCR expression analysis of CSF2 expression in mammospheres derived from either FVB wild type or 20wk old MMTV-Her2 pre-malignant mice. Technical replicates of three mice each are shown. Student's t-test was used for statistical analysis. c-e: Immunofluorescent staining (negative) of FVB wild type (c) and 22wk old MMTV-Her2 mammary glands (d) against CSF2 or with IgG control (e). Bars 25 mm. f: detection of CSF2 in overt MMTV-HER2 tumors (26-30 wks) supports that CSF2 is not detectable at protein level in early lesions. Bars 25 mm. g: Quantification of the mean fluorescence intensity for CCL2 staining in independent acini in Fig 6 e-g; n=2 independent experiment and multiple acini per group. h: quantification of the HER2 signal in Fig.6C, middle panel n=2 experiments. i: signal for CCR2 staining in the same tissues in Fig6c showing the signal in the whole fields and not only in selected areas exemplified in Fig6c. Bars 25 mm. j: qRT-PCR for CCL2 in the sorted cell populations indicated: HER2+, HER2+ early cancer cells; Neutro, neutrophils in the early lesion mammary glands; MACs, macrophages in the early lesion mammary glands; MONOs, monocytes in the early lesion mammary glands. k: quantification by ELISA of CCL2 secreted to the 24 hrs conditioned media of oncospheres produced by early cancer cells treated with control treatment (DMSO), lapatinib (LAP) or an IKK inhibitor (IKKi). l: qRTPCR for Wnt1 mRNA detected in RAW2647.7 monocytic cells treated with 20 ng per ml CCL2 for 24 hrs. All p values in all figure panels were calculated with Mann-Whitney test at 95% confidence.



Supplementary Figure 6. Analysis of human DCIS samples. Human DCIS sections were stained against CD68 and CD45 (a) and CD68 and cytokeratin 8/18 (b). b,c: Two individual ducts in one DCIS lesion of the same patient with either high E-Cadherin and low intra-epithelial macrophage (IEM) levels (c) or low E-Cadherin levels and high IEM levels (d) within the same patient. e: Quantification of E-Cadherin signal intensity in the region of cell junctions within ducts of the same patients with either high or low numbers of IEMs. f: Quantification of the frequency of macrophage infiltration in healthy adjacent and DCIS lesions separated by high and low grade subtype. Each point is a patient sample. g: Quantification of the frequency of macrophage infiltration in healthy adjacent and DCIS lesions separated by HER2 status. Each point is a patient sample. All p values in all figure panels were calculated with Mann-Whitney test at 95% confidence.

Supplementary table 1

Animal information for Fig.1

Figure	Experiment	Mouse strain	Age (weeks)	Mouse gender	Number of mice
Fig.1a)	H&E staining mammary gland	FVB	20	female	3-6
Fig.1b)	H&E staining mammary gland	MMTV-HER2	22	female	4-6
Fig.1c)	H&E staining tumor	MMTV-HER2	26-30	female	4-6
Fig.1d, 1g, 1j)	IF staining and quantification	FVB	20	female	4
Fig.1e, 1h, 1j)	IF staining and quantification	MMTV-HER2	14	female	3
Fig.1f, 1i, 1j)	IF staining and quantification	MMTV-HER2	22	female	5

Supplementary table 2

Animal information for Fig.2

Figure	Experiment	Mouse strain	Age (weeks)	Mouse gender	Number of mice
Fig.2a-c)	IF staining and quantification	MMTV-HER2	20	female	4
Fig.2d)	Quantitative PCR of mammary glands	FVB	20	female	4
		MMTV-HER2	20	female	7
Fig.2e-g)	IF staining and quantification	MMTV-HER2	24	female	3
Fig.2k,l)	Conditioned medium from primary mammospheres	FVB	20-22	female	2-3

Supplementary table 3

Animal information for Fig.3

Figure	Experiment	Mouse strain	Age (weeks)	Mouse gender	Number of mice
Fig.3b,c)	H&E staining mammary gland	MMTV-HER2	22	female	6-7
Fig.3d)	Quantitative PCR of mammary glands	MMTV-HER2	22	female	6-7
Fig.3e-g)	IF staining and quantification	MMTV-HER2	22	female	3
Fig.3h)	Circulating early cancer cells	MMTV-HER2	22	female	7
Fig.3i-k)	IF staining and quantification	MMTV-HER2	22	female	6-7

Supplementary table 4

Animal information for Fig.4

Figure	Experiment	Mouse strain	Age (weeks)	Mouse gender	Number of mice
Fig.4b)	Time until tumor onset	MMTV-HER2	23-38	female	9
Fig.4c)	Time until tumors were overt	MMTV-HER2	26-43	female	6-9
Fig.4d-f)	IF staining and quantification	MMTV-HER2	26-43	female	3-4
Fig.4g-i)	IF staining and quantification	MMTV-HER2	26-43	female	3-4
Fig.4j-l)	IF staining and quantification	MMTV-HER2	26-43	female	4-6

Supplementary table 5

Animal information for Fig.5

Figure	Experiment	Mouse strain	Age (weeks)	Mouse gender	Number of mice
Fig.5a-f, 5i-j)	CyTOF	FVB	20-24	female	5
		MMTV-HER2	14	female	5
		MMTV-HER2	22	female	5
Fig.5g,h, 5i-j)		MMTV-HER2	26-30	female	5
Fig.5k-n)	IF staining and quantification	FVB	20-24	female	3
		MMTV-HER2	24	female	3
		MMTV-HER2	26-30	female	3

Supplementary table 6

Animal information for Fig.6

Figure	Experiment	Mouse strain	Age (weeks)	Mouse gender	Number of mice
Fig.6a)	Western Blot with primary mammospheres	MMTV-HER2	20	female	3
Fig.6b)	Quantitative PCR of primary mammospheres	FVB	20-24	female	3
		MMTV-HER2	22	female	3
Fig.6c)	IF staining and quantification	FVB	20-24	female	3
		MMTV-HER2	22	female	3
Fig.6d)	OD for CCL2 via Elisa	FVB	20-24	female	2
		MMTV-HER2			
Fig.6e-g)	IF staining and quantification	MMTV-HER2	20-24	female	2

Fig.6h-j)	IF staining and quantification	MMTV-HER2	20	female	2
Fig.6k-m)	IF staining and quantification	MMTV-HER2	22	female	6-8
Fig.6n,o)	IF staining and quantification	MMTV-HER2	22	female	5

Supplementary table 6

Animal information supplementary Figures

Figure	Experiment	Mouse strain	Age (weeks)	Mouse gender	Number of mice
Sup Fig.1c-e)	IHC and quantification	MMTV-HER2	17	female	3
Sup Fig.2a)	IF and quantification	WT	11	female	1
Sup Fig.2a)	IF and quantification	MMTV-PyMT	11	female	1
Sup Fig.2b)	Flow cytometry	MMTV-HER2	22	female	3
Sup Fig.2c,d)	Flow cytometry	MMTV-HER2	20-24	female	3
Sup Fig.2e,f)	IF and quantification	MMTV-HER2	22	female	3
Sup Fig.2g,h)	Whole mount staining	MMTV-HER2	22	female	3
Sup Fig.3a)	IF and quantification	MMTV-HER2		female	2
Sup Fig.3b-f)	Flow cytometry	MMTV-HER2	26-43	female	3
Sup Fig.4a-c	CyTOF	FVB	20-24	female	5
		MMTV-HER2	20-24	female	5
Sup Fig.4d-g)	Flow cytometry	MMTV-HER2	20-24	female	3-5
Sup Fig.5b)	Quantitative PCR of mammospheres	FVB, MMTV-HER2	20	female	3
Sup Fig.5c-e)	Immunofluorescence	FVB, MMTV-HER2	22	female	3
Sup Fig.5f)	Immunofluorescence	MMTV-HER2	26-30	female	3
Sup Fig.5g)	IF staining and quantification	MMTV-HER2	20-24	female	2
Sup Fig.5h,i)	IF staining and quantification	FVB, MMTV-HER2	20-24	female	3
Sup Fig 5J	Qpcr ccl2	MMTV-HER2	18	female	4

Sup Fig.5K)	ELISA	MMTV-HER2		female	2
Sup Fig.5h,i)	IF staining and quantification	FVB, MMTV- HER2	20-24	female	3



Uncropped blots for the P-p65 and β -tubulin detection blots shown in Fig6A. Arrows point to the bands in Fig6A and the green boxes demark unrelated experiments.

